University of Alberta

The role of key nutrients during ETEC infection, development and immune function in newly weaned piglets

by

Ian Robert Johnson

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

in

Nutrition and Metabolism

Department of Agricultural, Food and Nutritional Science

Edmonton, Alberta

Fall 2004

Library and
Archives Canada

Published Heritage
Branch

395 Wellington Street
Ottawa ON K1A 0N4
Canada

Bibliothèque et
Archives Canada

Direction du
Patrimoine de l'édition

395, rue Wellington
Ottawa ON K1A 0N4
Canada

Your file Votre référence
ISBN: 0-612-95779-9
Our file Notre référence
ISBN: 0-612-95779-9

The author has granted a non-exclusive license allowing the Library and Archives Canada to reproduce, loan, distribute or sell copies of this thesis in microform, paper or electronic formats.

L'auteur a accordé une licence non exclusive permettant à la Bibliothèque et Archives Canada de reproduire, prêter, distribuer ou vendre des copies de cette thèse sous la forme de microfiche/film, de reproduction sur papier ou sur format électronique.

The author retains ownership of the copyright in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author's permission.

L'auteur conserve la propriété du droit d'auteur qui protège cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou aturement reproduits sans son autorisation.

In compliance with the Canadian Privacy Act some supporting forms may have been removed from this thesis.

Conformément à la loi canadienne sur la protection de la vie privée, quelques formulaires secondaires ont été enlevés de cette thèse.

While these forms may be included in the document page count, their removal does not represent any loss of content from the thesis.

Bien que ces formulaires aient inclus dans la pagination, il n'y aura aucun contenu manquant.

Canada

Acknowledgements

I have received assistance from numerous individuals during the course of this project, and I hope I do not forget any. First of all, thanks to my supervisor, Dr. Catherine Field. I would also like to thank my advisory committee members, which have included Dr. Ron Ball, Dr. Karen Madsen, Dr. Vickie Baracos, and Dr. Tom Clandinin. I would also like to thank Dr. Walter Dixon and Dr. Tapan Basu for their participation in my Candidacy Examination. Many of these individuals were instrumental in writing the numerous grants for this project, for which funding was obtained from the Alberta Agricultural Research Institutute, the Alberta Barley Commission, the National Sciences and Engineering Research Council of Canada and Degussa.

This project would not have been possible, without the excellent technical assistance of staff and students at the University of Alberta. Most of all, I would like to thank Ms. Sue Goruk. I have thoroughly enjoyed the summers spent at the Metabolic Unit Research Unit. I owe a special thank you to Brenda Tchir, Charlane Gorsak, Steve Melnyk and Clint Lysgaard, not only for their assistance, but also for providing a comfortable environment in which to conduct research. Thank you to Julie Laverdiere for her collaborative work with Ussing Chambers, as well as our discussions and paper swapping. Thanks to the rest of Dr. Madsen's Laboratory for their assistance and the use of these apparatus. Thanks to Dr. Vasanthan, Dr. Temelli and Arun Lehki for your assistance and input in the Beta-glucan project. I would also like to thank Jay, Janes, Diana, and the rest of the staff at the Swine Research Technology Centre for helping out. I have also received a great deal of assistance in the microbiology lab. Thanks to Dr. Mike Stiles and Dr. Lynn McMullen for your assistance in the early phase of this project. Thanks to Jennifer Snart, Denise Rivard and Dr. Gwen Allison for helping in the later phases of the project. I would also like to thank the staff of the AARI microbiology laboratory for your assistance. Thanks to Dorothy Rutkowski for your assistance at the flow cytometry unit. And last, but not least, a special thanks to all the summer students that have passed through our lab and done some of the project's grunt work: Megan, Arnie, Erin, Simirin, and Nicola.

I have also received a good deal of input from current and former students of Dr. Field. Thanks to Lindsay, Vera, Trish, Rose, and Megan. I should also thank Alexander Gaydamaka and Alfred Awere for their work in the early stages of this project. I would also like to recognize my other fellow graduate students and laboratory colleagues. Perhaps a disjointed group in such a diverse department, but it has been a pleasure getting to know students from many walks of life. Finally, I would like to add a special thank you to my non-academic acquaintances. To my parents, Phyllis and Dominic, and my sister, Laura, who have provided nothing but support, although the telephone was often their only means of communication. The best friends I have made in the city have been through various athletic endeavors. I have enjoyed the hard work involved with this project, but I appreciate the outlet and social network provided by these individuals. Finally, to Danielle, for keeping me up and being there during the end of my studies. The road less traveled was made more familiar thanks to your presence.

Table of Contents

Chapter 1 - INTRODUCTION

1.1 OVERVIEW **1**

PART I - RELEVANT CONCEPTS IN IMMUNOLOGY

1.2 THE IMMUNE SYSTEM AND ITS COMPONENTS **2**

- *A. Innate and Adaptive Immunitys* *2*
- *B. Gut-Associated Lymphoid Tissue* *3*

1.3 THE IMMUNE SYSTEM IN THE YOUNG ANIMAL **4**

- *A. Piglets and Neonatal Humans* *4*
- *B. Unique Charateristics of the Swine Immune System* *6*
- *C. Development of Piglet Innate Immune Function* *10*
- *D. Development of Piglet Peripheral Immune Function* *11*
- *E. Development of GALT in Piglets* *12*

PART II - RELEVANT CONCEPTS IN NUTRITION

1.4 PIGLET NUTRITION **13**

- *A. Protein and Amino Acids* *14*
- *B. Fiber* *15*

1.5 INTESTINAL EPITHELIUM AND NUTRIENT UPTAKE **16**

- *A. Intestinal Epithelium* *16*
- *B. Intestinal Functions* *17*
- *C. Assessment of Intestinal Function* *18*

1.6 IMMUNONUTRIENTS **19**

1.7 GLUTAMINE **19**

- *A. Glutamine and Intestinal Function* *21*
- *B. Immunological Influences of Glutamine* *22*
- *C. Swine Glutamine Metabolism* *23*

1.8 BETA-GLUCANS **24**

- *A. Beta-Glucan Metabolism* *24*
- *B. Immunological Influences of Beta-Glucans* *25*
- *C. Beta-Glutcan Supplementation in Siwine* *26*

PART III - INFECTION AND IMMUNE RESPONSE

1.9 ENTEROTOXIGENIC ESCHERICHIA COLI **27**

- *A. Escherichia coli infection and clinical diarrhea* *27*
- *B. Swine ETEC infections* *29*
- *C. K88 antigens* *30*
- *D. Cell Surface Characteristics* *31*
- *E. E. coli Infection Models* *33*

1.10 IMMUNE RESPONSE TO E. COLI INFECTION **33**

- *A. The Epithelial Barrier* *33*
- *B. Innate Immune Response to Infection* *34*
- *C. Adaptive Immune Response to Infection* *35*

1.11 CONCLUSIONS **38**

1.12 REFERENCES **48**

Chapter 2 - RESEARCH PLAN

2.1 RATIONALE **67**

2.2 OBJECTIVES AND HYPOTHESES **68**

2.3 CHAPTER FORMAT **71**

2.4 REFERENCES **72**

Chapter 3 - INFECTION TRIALS AND MODEL DEVELOPMENT

3.1 INTRODUCTION **74**

- *A. ETEC Models of Infection* *74*
- *B. In vitro and in vivo Surgical Models* *75*
- *C. In nivo physiological response to surgery and anesthesia* *75*
- *D. Effect of Surgery and anaesthesia on Immune Function* *76*
- *E. The systemic effect of inoculating E. coli into intestinal loops* *77*

3.2 ETEC INFECTION TRIALS (OBJECTIVE 1) **77**

- **Part I - Oral inoculation of newly weaned piglets with 3 doses of ETEC** **77**
- Materials and Methods *78*
  - Immunofluorescence *79*
  - Neutrophil Function *80*
- Results *82*
- Discussion *83*

Part II - Oral inoculation of newly weaned piglets with 2 high doses of ETEC ... *84*
Materials and Methods ... *84*
Results ... *84*
Discussion ... *85*

3.3 DEVELOPMENT OF SURGICAL MODEL (OBJECTIVE 2) ... **86**

Materials and Methods ... *86*
  Sample Collection ... *89*
  Immune Cell Isolation ... *90*
  Lymphocyte Proliferation ... *91*
  Ussing Chamber Measures ... *91*
Results ... *93*
Discussion ... *94*

3.3 DEVELOPMENT OF ETEC ADHESION ASSAY (OBJECTIVE 3) ... **95**

Materials and Methods ... *95*
Results ... *96*
Discussion ... *96*

3.4 SUMMARY AND CONCLUSIONS ... **97**

3.5 REFERENCES ... **112**

Chapter 4 - IMMUNE DEVELOPMENT OF PIGLETS FROM EARLY WEANING AT THREE WEEKS OF AGE TO FIVE WEEKS OF AGE

4.1 INTRODUCTION ... **115**

4.2 MATERIALS AND METHODS ... **117**

4.3 RESULTS ... **120**

4.4 DISCUSSION ... **123**

4.5 REFERENCES ... **148**

Chapter 5 - INFLUENCE OF GLUTAMINE ON IMMUNE AND GUT DEVELOPMENT AND ON THE EARLY STAGES OF ETEC INFECTION IN NEWLY WEANED PIGLETS

5.1 INTRODUCTION ... **152**

5.2 MATERIALS AND METHODS ... **155**

5.3 RESULTS ... **159**

5.4 DISCUSSION ... **162**

5.5 REFERENCES....192

Chapter 6 - INFLUENCE OF BARLEY-DERIVED BETA-GLUCANS ON SWINE IMMUNE AND GUT DEVELOPMENT

6.1 INTRODUCTION....199
6.2 MATERIALS AND METHODS....203
6.3 RESULTS....209
6.4 DISCUSSION....213
6.5 REFERENCES....243

Chapter 7 - SUMMARY AND FUTURE CONSIDERATIONS

7.1 SUMMARY....250
7.2 FUTURE CONSIDERATIONS....255
7.3 REFERENCES....262

Appendix

B-GLUCAN TRIAL FEED PROXIMATE ANALYSIS....263
INFECTION AND MODEL DEVELOPMENT ANTIBODY PROFILE....264
IMMUNE DEVELOPMENT AND GLN STUDY ANTIBODY PROFILE....265
B-GLUCAN STUDY ANTIBODY PROFILE....266
AMINO ACID PROFILE OF TEKLAD CASEIN....267
K88 ETEC GROWTH CURVE....268
ETEC ADHESION ASSAY SCHEMATIC....269
IMMUNE DEVELOPMENT AND GLN FEEDING DESIGN....270
B-GLUCAN FEEDING DESIGN....271

List of Tables

CLUSTER OF DIFFERENTIATION ANTIGENS AND THEIR FUNCTION 41

CYTOKINES AND THEIR FUNCTIONS AND SOURCES 42

ESSENTIAL AND NONESSENTIAL AMINO ACIDS 43

ION TRANSPORT PROPERTIES 44

INCIDENCE AND SEVERITY OF DIARRHEA IN ETEC-INOCULATED PIGLETS (3 CONCENTRATIONS) 97

INCIDENCE AND SEVERITY OF DIARRHEA IN ETEC-INOCULATED PIGLETS (2 CONCENTRATIONS) 97

USSING CHAMBER MEASURES OF ETEC-INOCULATED LOOPS 99

COMPOSITION OF BASAL DIET 132

INFLUENCE OF AGE ON NEUTROPHIL RESPONSE 133

INFLUENCE OF AGE ON PBL PHENOTYPES 134

INFLUENCE OF AGE ON MLN LYMPHOCYTE PHENOTYPES 135

INFLUENCE OF AGE ON PP LYMPHOCYTE PHENOTYPES 136

INFLUENCE OF AGE ON LYMPHOCYTE PROLIFERATION 137

INFLUENCE OF AGE ON PBL CYTOKINE PRODUCTION 138

INFLUENCE OF AGE ON MLN LYMPHOCYTE CYTOKINE PRODUCTION 138

COMPOSITON OF GLN TRIAL EXPERIMENTAL DIETS 173

WEIGHT AND FEED INTAKE OF GLN- AND CTL-FED PIGLETS 174

INFLUENCE OF GLUTAMINE ON NEUTROPHIL RESPONSE 175

INFLUENCE OF GLUTAMINE ON PBL PHENOTYPES 176

INFLUENCE OF GLUTAMINE ON MLN LYMPHOCYTE PHENOTYPES 177

INFLUENCE OF GLUTAMINE ON PP LYMPHOCYTE PHENOTYPES 178

INFLUENCE OF GLUTAMINE ON LYMPHOCYTE PROLIFERATION 179

INFLUENCE OF GLUTAMINE ON PBL CYTOKINE PRODUCTION 180

INFLUENCE OF GLUTAMINE ON MLN LYMPHOCYTE CYTOKINE PRODUCTION 180

INFLUENCE OF GLUTAMINE AND ETEC ON USSING CHAMBER MEASURES 181

COMPOSITION OF B-GLUCAN TRIAL EXPERIMENTAL DIETS 222

CHEMICAL COMPOSITION OF B-GLUCAN EXPERIMENTAL DIETS 223

WEIGHT, FEED INTAKE AND INTESTINAL MEASURES OF PIGLETS CONSUMING B-GLUCAN DIETS 224

BLOOD CHEMISTRY OF B-GLUCAN-FED PIGLETS 225
BLOOD IMMUNE CELL POPULATION CONCENTRATION OF B-GLUCAN-FED PIGLETS 226
INFLUENCE OF B-GLUCANS ON NEUTROPHIL FUNCTION 227
INFLUENCE OF B-GLUCANS ON PBL PHENOTYPES 228
INFLUENCE OF B-GLUCANS ON MLN LYMPHOCYTE PHENOTYPES 229
INFLUENCE OF B-GLUCANS ON PP LYMPHOCYTE PHENOTYPES 230
INFLUENCE OF B-GLUCANS ON LYMPHOCYTE PROLIFERATION 231
INFLUENCE OF B-GLUCANS ON PBL CYTOKINE PRODUCTION 232
INFLUENCE OF B-GLUCANS ON MLN LYMPHOCYTE CYTOKINE PRODUCTION 233
INFLUENCE OF B-GLUCANS ON USSING CHAMBER MEASURES 234
PROXIMATE ANALYSIS OF B-GLUCAN DIET INGREDIENTS 263
ANTIBODIES TESTED IN CHAPTER 3 264
ANTIBODIES TESTED IN CHAPTER 4 AND 5 265
ANTIBODIES TESTED IN CHAPTER 6 266
AMINO ACID PROFILE OF TEKLAD CASEIN 267

List of Figures

PATTERNS OF LYMPHOCYTE TRAFFICKING 45
USSING CHAMBER DIAGRAM 46
MOLECULAR BETA-GLUCAN STRUCTURE 47
PBL PHENOTYPES OF PIGLETS INOCULATED WITH 3 DOSES OF ETEC 100
NEUTROPHIL OXIDATIVE BURST FOLD INCREASE OF ETEC-INFECTED PIGLETS 101
PBL PHENOTYPES OF PIGLETS INOCULATED WITH ETEC 102
NEUTROPHIL OXIDATIVE BURST OF ETEC-INOCULATED PIGLETS 103
EFFECT OF SURGICAL PROCEDURE ON BLOOD PHENOTYPES 104
EFFECT OF SURGICAL PROCEDURES ON NEUTROPHIL OXIDATIVE BURST 105
EFFECT ON SURERY ON UNSTIMULATED LYMPHOCYTE PROLIFERATION 106
EFFECT OF SURGERY ON MLN LYMPHOCYTE PROLIFERATION 107
EFFECT OF SURGERY ON PBL PROLIFERATION 108
FLOW CYTOMETRY ANALYSES OF E. COLI BINDING 109
PHOTOGRAPH OF IN SITU SURGICAL PROCEDURE 110
PERCENT OF CELLS BINDING IN ETEC ADHESION ASSAY 111
INFLUENCE OF AGE ON PBL PHENOTYPES 139
INFLUENCE OF AGE ON PBL EXPRESSION OF CD45RA 140
INFLUENCE OF AGE ON MLN LYMPHOCYTE PHENOTYPES 141
INFLUENCE OF AGE ON MLN LYMPHOCYTE EXPRESSION OF CD45RA 142
INFLUENCE OF AGE ON PP LYMPHOCYTE PHENOTYPES 143
INFLUENCE OF AGE ON PP LYMPHOCYTE EXPRESSION OF CD45RA 144
INFLUENCE OF AGE ON PBL & MLN LYMPHOCYTE PROLIFERATOIN TO PHA 145
INFLUENCE OF AGE ON PBL & MLN LYMPHOCYTE PROLIFERATOIN TO PWM 146
INFLUENCE OF AGE ON PBL & MLN LYMPHOCYTE PROLIFERATOIN TO LPS 147
INFLUENCE OF GLUTAMINE ON PBL PHENOTYPES 182
INFLUENCE OF GLUTAMINE ON PBL EXPRESSION OF CD45RA 183
INFLUENCE OF GLUTAMINE ON MLN LYMPHOCYTE PHENOTYPES 184

INFLUENCE OF GLUTAMINE ON MLN LYMPHOCYTE EXPRESSION OF CD45RA 185
INFLUENCE OF GLUTAMINE ON PP LYMPHOCYTE PHENOTYPES 186
INFLUENCE OF GLUTAMINE ON PP LYMPHOCYTE EXPRESSION OF CD45RA 187
INFLUENCE OF GLUTAMINE ON MLN LYMPHOCYTE PROLIFERATOIN 188
INFLUENCE OF GLUTAMINE & ETEC ON JEJUNAL MANNITOL PERMEABILITY 189
INFLUENCE OF GLUTAMINE & ETEC ON JEJUNAL SHORT-CIRCUIT CURRENT 190
INFLUENCE OF GLUTAMINE & ETEC ON JEJUNAL POTENTIAL DIFFERENCE 191
FLOW CYTOMETRY ANALYSIS OF ETEC ADHESION ASSAY 235
INFLUENCE OF B-GLUCANS ON BLOOD CHEMISTRY 236
INFLUENCE OF B-GLUCANS ON PBL EXPRESSION OF CD45RA 237
INFLUENCE OF B-GLUCANS ON MLN LYMPHOCYTE EXPRESSION OF CD45RA 238
INFLUENCE OF B-GLUCANS ON PP LYMPHOCYTE EXPRESSION OF CD45RA 239
INFLUENCE OF B-GLUCANS ON ILEAL MANNITOL PERMEABILITY 240
INFLUENCE OF B-GLUCANS ON ILEAL CONDUCTANCE 241
INFLUENCE OF B-GLUCANS ON ETEC BINDING INTENSITY 242
K88AC ETEC GROWTH CURVE 268
DIAGRAM OF ETEC ADHESION ASSAY 269
EXPERIMENTAL DESIGN FOR DEVELOPMENT AND GLUTAMINE TRIALS 270
EXPERIMENTAL DESIGN FOR B-GLUCAN TRIALS 271

List of Abbreviations

Average Daily Gain - ADG

Beta-Glucans – B-glc

B lymphocytes – B cells

Body Weight - BW

Bovine Serum Albumin - BSA

Cluster of Differentiation - CD

Complete culture media - CCM

Conductance - G

Control - ctl

Cyclic 3^1,5^1-guanosine monophosphate - cGMP

Cyclic 3^1,5^1-adenosine monophosphate - cAMP

Cytotoxic/Suppressor T lymphocytes - Tc

Dihydrorhodamine – DHR

Dimethyl sulphoxide - DMSO

Escherichia coli – E. coli

Enterotoxigenic Escherichia coli – ETEC

Ethylenediaminetetraacetic acid - EDTA

Feed Intake – FI

Fetal Calf Serum - FCS

Forskolin - fors

Fluorescein isothiocyanate - FITC

Glutamine – gln

Gut Associated Lymphoid Tissue – GALT

Hank's Balanced Salt Solution - HBSS

Helper T lymphocytes – Th

Immunoglobulin – Ig

Interleukins - IL

Lipopolysaccharide – LPS

Mannitol Permeability - ManP

Mesenteric Lymph Nodes - MLN

Peripheral Blood Lymphocytes – PBL

Peyer's Patches - PP

Phorbol myristate acetate - PMA

Phorbol myristate acetate/ionophore – PMAI

Phosphate Buffered Saline - PBS

Phycoerythrin – PE

Phytohemagglutinin - PHA

Pokeweed mitogen - PWM

Polymorphonuclear Neutrophils – neutrophils

Potential Difference - PD

Reactive Oxygen Intermediates - ROI

Short-chain fatty acid – SCFA

Short-circuit Current - Isc

T lymphocytes – T cells

Wild type - WT

Chapter 1 – INTRODUCTION

1.1 Overview

Defining the optimal immune health of an organism has proven difficult and there is not a great deal established, particularly in livestock species such as swine. This thesis will examine the immune health of swine, specifically young piglets during the stressful weaning period. The importance of nutrition and immunology has grown as researchers continue to look for new ways of improving health status and the newer concepts of health prevention and promotion gain prominence. Although the relationship between nutrition and immunology was formally recognized in the 1970s when immunological measures were first introduced as an integral component of assessing nutritional status, the past decade has resulted in an explosion of new knowledge in this area. What has become apparent is that nutritional state and specific nutrients impact on the immune system directly, and conversely, altered immune status can impact on nutritional status. The evidence that has accumulated indicates that the beneficial effects of nutrients extend beyond preservation of vital functions such as growth and maintenance. In fact, a great deal of support has been documented that pharmacological levels of several nutrients may provide optimal health benefits. The clinical use of such nutrients has been referred to as immunonutrition. This chapter will provide an introduction of the interrelationships between the rapidly growing fields of nutrition and immunology. Included here will be information relating to immunonutrition, a description of the swine immune system, *Escherichia coli* (*E. coli*) infection and the immune response to infection.

Part I - Relevant Concepts in Immunology

1.2 The Immune System and its Components

Innate and Adaptive Immunity

Innate immune defenses are generally described as the nonspecific components of the immune system that function without depending upon prior exposure to a particular pathogen, and that do not change to any great degree in response to a particular infection. Macrophages and their precursors, monocytes and polymorphonuclear neutrophils (neutrophils), make up the major cellular components of the innate immune system. This component of the immune system provides the early phases of host defense that protect the organism during the 4-5 days it takes to mount a specific immune response. The induction of a specific immune response, such as the production of antibodies to a particular pathogen, is part of the adaptive or acquired immune response. Inherent to the specific response are lymphocytes, which differ in expression of cell-surface antigens (Cluster of Differentiation (CD) antigens, see Table 1) and circulate between blood and lymphoid tissues. The complex interactions among immune cells are mediated by a group of secreted low-molecular weight proteins that are collectively designated cytokines to denote their role in cell-to-cell communication. A list of cytokines with their associated functions is given in Table 2. The adaptive response can further be divided into the humoral immune system, which is important in defense against bacterial or other extracellular antigens, and the cellular immune system (B and T cells), which is important in defense against intracellular pathogens such as viruses. Thymocytes surviving the selection process eventually exit the thymus and migrate to the peripheral lymphoid organs, which include the spleen, which filters antigens within the bloodstream, lymph nodes, which filters afferent lymph, and mucosal tissues, such as the intestinal, respiratory and urogenital tracts, which sample luminal antigenic substance via a variety of mechanisms (Gaskins 1998).

Gut-associated lymphoid tissue

The total mucosal surface area in adult humans is approximately 200-400 m^2 and is protected by the gut-associated lymphoid tissue (GALT) (reviewed in (Langkamp-Henken et al. 1992)), making it the largest immune organ of the body. Within GALT are found both nonaggregated and aggregated lymphoid cells.

The nonaggregated components of GALT consist of lamina propria (LP) lymphoid cells (diffuse population of T helper cells (Th; CD4), B lymphocytes (B cells), and plasma cells) and intraepithelial lymphocytes (IELs - predominantly CD8 T suppressor cells). The majority of the antibody-forming cells found within the lamina propria produce and secrete Immunoglobulin (Ig) A (approximately 90% (Laissue et al. 1993), although IgM-, IgG-, IgD-, and IgE-producing plasma cells are also found in this region (Langkamp-Henken et al. 1992). Mucosal mast cells and intestinal macrophages found interspersed within the vasculature and lymphatic-rich lamina propria of the intestine are also components of the nonaggregated lymphoid tissue (Langkamp-Henken et al. 1992).

The aggregated components of GALT consist of isolated lymphoid follicles, the appendix, and the GALT components that will receive a great deal of attention in this thesis, the Peyer's patches (PP) and mesenteric lymph nodes (MLN). MLNs play a pivotal role in the maturation of lymphocytes, contain a large number of IgA precursor B cells and are located on the route from the priming site back to the intestinal mucosa (McWilliams et al. 1977). They receive antigens from the small and large intestine (antigen processing reviewed in (Keren 1992)) via lymphatics running along the mesenteric arteries. Thus large numbers of lymphocytes emigrate from the PP and reach the blood after expansion and maturation within the mesenteric lymph nodes (reviewed in (Rothkotter et al. 1999)). PPs are present throughout the small intestine, but are most prominent in the ileum (Keren 1992), the domed surface of which can be found protruding into the intestinal lumen from between villi. They consist of distinct regions: the dome (immediately beneath the epithelium), the follicles (which vary from 5 to more than 900 per patch (Cornes 1965) and the thymus-dependent area between the follicles. Circulating blood lymphocytes become compartmentalized when they immigrate into PP by high endothelial venules (HEV) (Rothkotter et al. 1999). In HEV the immigration of cells involves several steps, beginning with rolling along the endothelium, followed by attachment and finally transmigration of the activated cells through the wall of the vessel (Springer 1994). PP follicles contain mostly B cells, whereas the interfollicular area and the above-dome region contain predominantly T lymphocytes (T cells) and the dome region is rich in plasma cells, macrophages, and dendritic cells (Keren 1992; Langkamp-Henken et al. 1992).

1.3 The Immune System in the Young Animal

Piglets and Neonatal Humans

Similar to piglets, infants are born with some degree of immunological immaturity affecting both antibody and cell-mediated functions, which are proposed to contribute to their increased susceptibility to gastrointestinal infections (Crockett 1995; Kovarik & Siegrist 1998; Mannick & Udall, Jr. 1996). Additionally, weaning in infants is associated with an increased incidence of gastrointestinal infections. Some possible contributors include: the absence of immune factors provided by breast milk (Eglinton et al. 1994; Skansen-Saphir et al. 1993), the immaturity of the infant's gut and gut associated immune defenses (Eglinton et al. 1994; Mannick & Udall, Jr. 1996), the high proportion of immature T cells and the predominantly Th2 type cytokine response (Bjorksten 1999).

Swine are being used as an animal model in experimental medicine because of physiological similarities to infants (described in (Moughan et al. 1992; Wykes et al. 1993)). This species represent a convenient, easily available, relatively economical model and possesses several advantages compared to mice, which have a very short gestation and a distance in phylogeny. Piglets possess similar nutritional requirements, intestinal physiology and metabolism with the human infant. The full-term neonatal piglet has a body composition similar to that of the premature infant (23-31 wk gestation), permitting more invasive methods of investigation while maintaining clinical relevance. However, a human infant doubles its birth weight by 4-6 months, whereas a piglet doubles its birth weight in 7-10 d, but this provides a rapid model of growth and development for focused investigations. Regulated mating, high fecundity and the size of fetuses are additional advantages of this experimental model. In the case of gut immunology the pig offers attractive opportunities as an experimental model in a number of areas including: immunopathogenesis of bacterial and viral enteric diseases, mucosal vaccination, small bowel transplantation, food hypersensitivity and the study of the influence of maternal factors upon immune development. The ability to isolate large numbers of cells following disruption of solid organs, to cannulate gut lymphatics and to provide a natural enteric disease model in piglets offers considerable advantages for the study of this compartment of the immune system (Stokes et al. 1994). However, much remains to be determined about the function of the swine immune system. For example, compared to humans and mice less is understood about cytokine functions in swine, partly attributed to a limited number of commercially available biological reagents.

Unique Characteristics of the Swine Immune System

While there are many similarities between the porcine and human immune system, there are also important differences. Perhaps the most important trait of the immune system of piglets, especially from a commercial and health perspective, are differences in innate immune function at birth. While the newborn infant is born with antibodies from their mothers for protection against infections (reviewed in (Glezen 2003)), the piglet is born with none. The multi-layered placenta of the pig prevents the transit of maternal immunoglobulins into the fetus (Pabst & Rothkotter 1999) and also prevents access by most microorganisms (Klobasa et al. 1986). Therefore this sheltered environment of the fetal immune system is free of antigen-driven differentiation and can develop basically unbiased by external immunomodulatory factors (Sinkora et al. 2002). In contrast to rodents and humans, pig intestinal epithelial cells do not express MHC Class II molecules, but they are present on a number of other cell types in the subepithelial lamina propria (a full description of antigen presenting cells is given in (Stokes et al. 1996)).

The T lymphocyte composition of swine are one of the most diversified T lymphocyte population in any species (reviewed in (Saalmuller et al. 1989)). Pigs have been shown to have four T-lymphocyte subsets in their peripheral blood and lymphoid tissues. In addition to the classical phenotypes of CD4+CD8- helper/inducer T cells (Th) and CD8+CD4- cytotoxic/suppressor T cells (Tc), there is a double negative CD4-CD8- T cells subset which contains the majority of γδ T cells, and a unique double positive CD4+CD8+ T cell subset, containing memory αβ T cells. There is also normal co-expression of the CD2 marker with either the CD4 or CD8 antigen. Thus greater than 90% of peripheral T cells express the CD4 and/or CD8, as well as the CD2 antigen (lymphocyte populations of swine detailed in (Lunney & Pescovitz 1987)). The CD4-CD8- subpopulation is unique for its γδ T cell receptor, and further consists of CD2+ and CD2- subsets, which represent a large percentage of lymphocyte T cells in swine, but not in humans and rodents (Becker & Misfeldt 1993).

Additional information about the unique CD4+CD8+ double positive T cell population is necessary, since their very nature is inconsistent with the generalization that the expression of the CD4 and CD8 cell surface glycoproteins is mutually exclusive in extrathymic lymphocytes (Saalmuller et al. 1989). Double positive T cells (reviewed in (Lunney & Pescovitz 1987; Saalmuller et al. 1987b)) can be found in peripheral blood and secondary lymphoid organs, but the variation in the proportion of these cells in blood is high (8-60% (Saalmuller et al. 1987b)). In other species, such as humans, CD4-CD8- precursor cells differentiate during thymic ontogeny into CD4+CD8+ thymocytes, which, upon further differentiation, lose either CD4 or CD8 giving rise to the mature phenotypes CD4-CD8+ and CD4+CD8- (Saalmuller et al. 1987b). Both the CD4 and CD8 T cell populations are known to have specific functions, which leads to questions concerning the purpose of simultaneous expression of these antigens. Dual expression of these two co-receptors must have an effect on their function and ability to recognize antigen, however, their exact function is not known (Pescovitz et al. 1994). What is known is that based on the expression of cell surface antigens and the size of the cells, double positive T cells are thought to belong to a mature resting population of lymphocytes (Pescovitz et al. 1990; Saalmuller et al. 1987a; Saalmuller & Bryant 1994). It is thought that this double positive population are CD4+ Th that have acquired and retained the CD8 antigen during development (Patel et al. 1989; Saalmuller et al. 1987b; Zuckermann & Gaskins 1996). This is supported by the fact that their percentage starts very low (<2% in at 3 d) and gradually increases (up to 16% (Pescovitz et al. 1994)) over the first year of life (Summerfield et al. 1996; Zuckermann & Husmann 1996).

Another characteristic of swine T cells is that in comparison with humans, there is a different proportion of CD4 and CD8 cells in peripheral blood lymphocytes (PBL) (Lunney & Pescovitz 1987). In peripheral blood, the pig normally has 25% CD4+ T cells and 40% CD8+ T cells resulting in a 0.60 CD4/CD8 ratio compared to 1.5-2.0 for humans (Pescovitz et al. 1985). The low CD4/CD8 ratio reported in swine is only found in humans suffering from HIV infections (reviewed in (Rosenberg et al. 1998). It is not known why these species differ in their T cell proportions, nor is it clear if these unique T cell proportion and phenotypes offer any particular advantage to the piglet.

Differences also are reported in the lymphocyte migration patterns of piglets compared to other species (Figure 1)(reviewed in (Rothkotter et al. 1999)). In contrast to other species, pig lymph nodes are actually inverted with a dense medulla lacking sinuses and cords (Binns 1982). Lymphocytes leave these tissues directly via blood capillaries rather than in efferent lymph (Bennell & Husband 1981; Binns 1982). The reasons for these differences in morphology are not known, but might results in activated lymphocytes being able to enter the circulatory system more quickly to enable them to home back to mucosal sites more quickly to provide local protection.

An additional anatomical species-specific difference exists in the distribution of PPs along the small intestine. In humans, discrete PP up to ~ 10 cm in length is distributed throughout the small intestine and have increased numbers in the ileum (reviewed in (Barman et al. 1997)). Swine have three structurally, functionally and developmentally different types of PP; the jejunal PP, the ileal PP and ten irregular spiral PPs in the colon (detailed in (Binns 1982; Pabst et al. 1988; Stokes et al. 1996)). The jejunal PPs are distributed along the jejunum and proximal ileum, are approximately 25-35 in number, comparatively small, and persist throughout life. Although there is a developmental increase in the size of Peyer's patches, the number and the position of individual patches remains constant. In contrast, the ileal PP is one very large ileocecal patch which may extend for up to 2.5 m along the terminal ileum but which involutes at about 1year of age. These differences in the morphology of jejunal and ileal PP are apparent only during a short period of postnatal life and are affected by luminal antigenic challenge (the influence of antigen is detailed in (Rothkotter et al. 1999)). This was demonstrated by the vast number of B cells found in conventional animals (at 1.5 months) compared to germfree pigs where many more T cells are found (Rothkotter & Pabst 1989). PP in the jejunum and ileum were also shown to differ not only in structure, but also lymphocyte migration and production (Pabst et al. 1988). Similar to sheep, the ileal PP is a primary B-cell organ because it contains more than 90% B cells, compared to 35% MLN and 45% in the jejunal PP (Andersen et al. 1999).

Development of Piglet Innate Immune Function

Phagocytic cells such as macrophages are the main cells type engaged in protection against infection in the first few weeks following birth (Tlaskalova-Hogenova et al. 1994). Previously, no significant age-related changes were observed in polymorphonuclear neutrophil (PMN) function in newborn piglets compared to piglets at 6 weeks of age (Hoskinson et al. 1990). This is influenced by luminal contents, as germ-free piglets have been shown to have low numbers of PMNs (Travnicek et al. 1989) that were increased substantially by intestinal monoassociation with bacteria or by killed bacterial suspensions given intraperitoneally (Tlaskalova-Hogenova et al. 1994).

Development of Piglet Peripheral Immune Function

Peripheral immunity of piglets continues to mature during the first few weeks after birth, and is further influenced by the weaning process. Although the proportion of circulating B cells of the newborn piglet is similar to that in adults, the total number in lower in the first 10 postnatal days (McCauley & Hartmann 1984). Weaning has been shown to transiently increase the concentration of peripheral B cells (McCauley & Hartmann 1984). Adult numbers of IgA+ B cells are reached by 4 weeks of age, while IgM+ cell numbers continue to increase until 3-4 months of age (Brown & Bourne 1976; Rothkotter et al. 1991). T lymphocytes have been shown to develop with relatively equal percentage of CD4+ and CD8+ peripheral lymphocytes as the animals mature (Jonjic et al. 1987). Corresponding with the substantial amount of antigen exposure at weaning, the number of T cells rises to values significantly higher than in adults by 12 days after weaning (McCauley & Hartmann 1984). During their first few weeks, piglet peripheral blood lymphocytes are able to respond (^{3}H-thymidine incorporation) to T cell mitogens (phytohemagglutinin (PHA) and ConA)(Hoskinson et al. 1990; Valpotic et al. 1989). Early weaning of piglets (before 5 wks) has also been shown to have a suppressive effect on peripheral blood lymphocyte response to PHA (Blecha et al. 1983) and a hyper-response (anti-soy IgG) to soy protein dietary antigens (Bailey et al. 1993; Miller et al. 1984).

Development of GALT in Piglets

At birth, a detectable number of immature B cells are present in the lymph nodes and Peyer's patches of piglets (Bianchi et al. 1992; Pabst & Rothkotter 1999). However, between days 1 and 42, there is a large increase (15-fold) in the cell concentration in the piglets ileal PP (Stokes et al. 1996) and the size of PP has been shown to increase several-fold following birth (Chu et al. 1979). In GALT, weaning is followed by a brief decline in the number of CD4+ cells (d 1), combined with an expansion of CD8+ cells (d2 and d4) resulting in a decreased ratio of CD4+/CD8+ T cells (Spreeuwenberg et al. 2001).

There is also an increase in CD2+ T cells and increased macrophage/granulocyte cells in the proximal small intestinal villi and crypts (Vega-Lopez et al. 1995). The immune cell composition of PP also changes as the animals mature, with a higher concentration of T cells in the jejunal than ileal PP (Barman et al. 1997). The ileal PP are very high in B cells (63.1%) and a low proportion of T cells (6.3%) (Rothkotter & Pabst 1989). As piglets mature, there are also increases in both CD4+ and CD8+ numbers in PP (Pabst et al. 1988; Rothkotter & Pabst 1989). Piglet MLN contain antigen-sensitized mucosal lymphocytes that have migrated from the PP and are rich in Ig+ lymphocytes and CD4+ cells (Rothkotter et al. 1993; Salmon 1987). MLN cell composition appears to be relatively stable during postnatal development (Whary et al. 1995). Functionally, B cells increase in an isotype-specific manner with increasing antigen exposure after birth (Klobasa et al. 1981), with IgM+ cells being synthesized first (2 weeks), followed by synthesis of IgA (3 weeks) and IgG (5 weeks).

Part II - Relevant Concepts in Nutrition

1.4 Piglet Nutrition

Within the weeks following birth, the steadily increasing nutrient demands of the rapidly growing young piglets can no longer be met through sow's milk (piglet nutrition reviewed in (Patience & Thacker 1989)). As a result, less expensive, pre-starter diets based on high energy grains (i.e. corn, wheat, barley), readily digested protein supplements such as soybean meal, and additional ingredients (increase palatability and digestibility) are fed to piglets. This process is often referred to as weaning and although the age of piglet weaning can vary, intensive agriculture practices have resulted in much earlier weaning of piglets. This stressful period, when young piglets are suddenly forced to rely on foreign sources of nutrients often results in a decrease in growth rate and an increased susceptibility to infections (Patience & Thacker 1989). The importance of nutrition at this time is exemplified by a high level of preweaning mortality (25-30%) that is observed in commercial swine production operations (Patience & Thacker 1989).

Antibiotics have played a major role in the growth and development of the swine industry for over three decades. The ability of animicrobials to increase growth rate, improve feed utilization, and reduce mortality from clinical disease has been well documented (reviewed in (Cromwell 2002)). The improvement in growth and feed efficiency resulting from the addition of antimicrobial compounds to starter diets is very impressive and on average, a 12-15% improvement in daily gain and a 5-6% improvement in feed efficiency can be expected as a result of supplementing swine starter diets with antibiotics (Patience & Thacker 1989).

The pig's nutrient requirement, which is the amount of a given nutrient required by the pig to maximize performance, must be established to properly balance swine diets. Nutrient requirements for young piglets are more completely described elsewhere (NRC 1998). More recently, nutrition research in the piglet is becoming oriented towards optimizing health and not simply promoting growth. Before continuing, a brief description of the two macronutrients of interest in this thesis will be provided.

Protein and Amino acids

A great deal of emphasis in swine nutrition is on amino acid requirements (reviewed in (Pencharz & Ball 2003)). When a less than ideal mixture of essential amino acids is fed, protein synthesis is reduced, and net protein accretion and nitrogen balance is lower. An essential amino acid is that which cannot be endogenously synthesized by using carbon skeletons or other metabolites and amino groups derived from other amino acids in sufficient amounts and therefore must be obtained by the organism in the diet. During growth several amino acids are regarded as conditionally indispensable, when these must be obtained in the diet until the defined growth period has passed (reviewed in (Pencharz & Ball 2003). Likewise, during immunocompromised states, specific amino acids are required in increased amounts to support the immune response and increased physiological needs in these conditions (reviewed in (Field et al. 2000a)). An example of a conditionally essential amino acid is glutamine (gln), which will be discussed in detail in subsequent sections. Amino acids along with studies examining their requirements are given in Table 3.

Fiber

In addition to providing excellent sources of energy and protein, many plant-based feed crops have a component that is resistant to hydrolysis by mammalian enzymes and therefore cannot be digested and absorbed by the animal. Dietary fiber is traditionally grouped according to fermentability or solubility. Based on solubility, fibers are classified as either neutral detergent fiber (NDF), an estimate of the total plant cell wall consisting primarily of cellulose, hemicellulose, and lignin, or as acid detergent fiber (ADF), which eliminates the hemicellulose content to estimate cellulose and lignin only. Utilization of fiber by nonruminants has been shown to vary considerably (0 to 97 percent (Rerat 1978)) depending on the fiber source, degree of lignification, level of inclusion, extent of processing, age and weight of the animal, as well as the individual variation that exists among pigs (NRC 1998). Fiber Sources with high water-holding capacity (i.e. gums or pectins) tend to be more readily degraded or fermented by microbes in the large bowel (Schneeman 1999). Polysaccharides entering the large bowel are the primary substrates for fermentation and result in growth of the microflora or an increased microbial mass (Schneeman 1999). The products of fermentation, which provide the only nutritional value of dietary fiber, include CO_2, H_2, and short-chain fatty acids (SCFA). The SCFA (acetate, propionate and butyrate) are used as an energy source and have been shown to promote the health of the intestinal mucosa (reviewed in (Velazquez et al. 1997)). A major constituent of dietary fibers are the beta-glucans (B-glc) isoforms (Sundberg et al. 1996), which will be discussed in detail in a subsequent sections.

1.5 Intestinal Epithelium and Nutrient Uptake

Intestinal Epithelium

The structure and function of intestinal epithelial cells are vitally important in the process of nutrition uptake (intestinal epithelial function reviewed in (McKay & Perdue 1993)). These cells rest on a basement membrane and are connected to each other by actin-rich tight junctions and looser desmosomes, located in the uppermost third of the cell, which maintain the continuity of the epithelium. The importance of epithelial tight junctions in permeability will be described in a subsequent section. The apical surface of the enterocyte is folded into numerous microvilli that allow for a 600-fold increase in intestinal surface area. The epithelium can be divided into three functionally separate regions – the crypt, maturation zone and villus. Enterocytes originate from stem cells in the crypt, migrate to the villus epithelium, and after a life span of 4-5 days, are sloughed off into the lumen from the extrusion zone at the villus tips and are subsequently digested and absorbed. During migration, the individual cells differ in their absorptive and secretive capacity as well as their content of digestive enzymes (i.e. sucrase, maltase). Thus the migration of enterocytes ensures that the mature cells receive maximum exposure to the contents of the intestine while the immature cells are protected from the physical forces of food moving along the length of the intestine. A complex structural feature that covers epithelial cells is the mucus layer, consisting of mucin molecules and large amounts of associated glycoproteins and glycolipids (Conway et al. 1990; Laux et al. 1986). Although mucin appears to make up only a small percentage of the mucin layer, its' heavily glycosylated subunits (ranging in size from 250 to 500 kDa) is responsible for the gel-like properties of the mucus layer (Conway et al. 1990). Tight junctions (zonula occludens) are the apical epithelial intercellular junctions responsible for maintaining the integrity of the epithelial barrier. Tight junctions can either act as a tight seal, enabling the epithelium to establish and maintain steep gradients of concentration and/or electrical potentials and prevent waste products or other toxic substances from entering the body fluids or may be relatively 'leaky', providing an important pathway for passage of ions and small solutes across the epithelium through the paracellular pathway (Nusrat et al. 2000). Tight junctions maintain the distinctive polarized microenvironments on both sides of the cell, which is absolutely vital if the epithelium is to achieve transport in a particular direction (Nusrat et al. 2000).

The nervous system also influences intestinal epithelial function. The effector systems in the gut are controlled by the three divisions of the autonomic nervous system: the sympathetic, parasympathetic, and enteric systems. A complete description of neurotransmitter regulation of ion transport in the porcine intestinal tract has been published previously (Brown & O'Grady 1997). Neurotransmitters of the enteric nervous system control most of the intestinal effector systems, including intestinal ion and fluid transport, by activating submucosal afferent and efferent neurons that innervate the epithelial cells. Intestinal ion transport is also incfluenced by protein kinases and changes in the levels of intracellular mediators such as: cyclic $3^1,5^1$-adenosine monophosphate (cAMP), cyclic $3^1,5^1$-guanosine monophosphate (cGMP),. Ca2+, phosphosinositides, diacylglycerol and arachidonic acid metabolites.

Intestinal Function

In an adult human, approximately 9 L of fluid enters the upper small intestine per day and an additional 1 L is secreted by the small intestine. Much of this secretion is driven osmotically by the active secretion of anions, especially chloride (Cl-) (Keely & Barrett 2000), which is regulated both inter- and intracellularly. Intercellular regulation is achieved via the actions of hormones, neurotransmitters, and other mediators that are released in the local environment of the epithelium and subsequently bind to epithelial receptors. Intracellular regulation, which can be further subdivided to those responses that promote Cl- secretion and those that limit or terminate it, are those signaling pathways that link receptor occupancy to the downstream response of Cl- secretion (Keely & Barrett 2000). In general, Cl- secretion is activated either by increases in the levels of cAMP or cGMP or via elevations in cytoplasmic calcium concentrations. Cyclic nucleotides evoke a sustained increase in Cl- secretion, whereas the calcium-dependent response is transient (Barrett 1993). Changes in levels of these intracellular messenger levels ultimately leads to phosphorylation of specific Cl- channels (Uzzau & Fasano 2000) thus enabling Cl- secretion. Many pathogens can induce Cl- secretion, leading to massive secretion of water and electrolytes and copious diarrhea by exploiting these host signallings using specific toxins that affect different steps of the cellular control of Cl- secretion (Uzzau & Fasano 2000).

Since only a relatively small amount of fluid (1-1.5 L) enters the colon, the small intestine is responsible for absorption of several liters of isotonic fluid, in addition to ingested nutrients and may approach up to 12 L/day. For proper intestinal function, the gut must acquire and excrete the major inorganic ions (Na^+, K^+, Cl^-, HCO_3^-). These ions effectively define the sizes of the intracellular and extracellular body fluid compartments and, by being unequally distributed between the two, support a number of important physiological processes (reviewed in (Holtug et al. 1996)). However, in order to cross the polarized epithelial cell, large solutes that carry a net charge must be actively transported because of the additional thermodynamic effect of moving the charge across any difference in electric potential that exists between the solutions on the two sides of the membrane (reviewed in (Ussing 1994)). The transport properties of the major inorganic ions are given in Table 4.

Assessment of Intestinal Function

Intestinal function can be assessed with many different techniques, including those which have been developed *in vivo* and those performed *in vitro*. By using *in vitro* techniques it is possible to control intrusive factors present under *in vivo* conditions, study exactly defined segments of the bowel, expose mucosal tissue to various drugs without incurring problems with drug toxicity and closely monitor transepithelial transport. These techniques also offer advantages in comparison to some cell culture models. For examples, while the Caco-2 cell line exhibits many functional characteristics of the human small intestinal enterocyte, it was still created by carcinogenic transformation and is thus non-normal in several ways. The classical experiments in the 1940s and 1950s carried out by Hans Ussing and his colleagues led to the development of active versus passive transport theories and also the development of methods and apparatus still used by electrophysiologists [The Ussing chamber technique (first decribed in 1951 (Ussing &

Zerahn 1951), subsequently in (Söderholm 1998)(Albin & Tappenden 2001)) shown in Figure 2].

1.6 Immunonutrients

Nutrition research has evolved beyond meeting daily requirements for proper functioning, to include optimal research for proper metabolic function. Among these, is nutritional immunology, which seeks to find and establish the mechanism by which specific nutrients can influence immune function. Many nutrients have been shown to possess immune-enhancing properties, including lipids, specifically n-3 fatty acids, vitamins, such as vitamin A, vitamin E, vitamin C, minerals, such as zinc, selenium, copper, and iron (reviwed in (Field et al. 2002)). Although the importance of each of these nutrients should not be understated, the remainder of this section will describe in detail the two nutrients of interest, gln and B-gluc.

1.7 Glutamine

The essentiality of the amino acid gln has also been demonstrated in renal ammoniagenesis and as a precursor for the biosynthesis of a number of important compounds such as nucleotides (Krebs et al. 1980), NAD^+, amino sugars, proteins (Brand et al. 1989; Szondy & Newsholme 1989) and other amino acids (including proline and arginine (Wu & Meininger 1993)). After the transport of cytoplasmic gln into the mitochondrial matrix, it is cleaved by glutaminase to yield glutamate and ammonia (Bode & Souba 1999), the glutamate being utilized as a gluconeogenic substrate upon conversion to 2-oxoglutarate and entry into the TCA cycle (Bode & Souba 1999).

Therefore, rapidly growing cells, such as intestinal mucosal cells (Ardawi & Newsholme 1985; Windmueller & Spaeth 1974), stimulated cells of the immune system (Ardawi & Newsholme 1983; Brand et al. 1989; Wu et al. 1992; Wu et al. 1991a; Wu et al. 1991b) can use gln as a primary oxidative fuel. Increasing evidence indicates that gln can also play a crucial role in osmotic regulation of cell volume and induce phosphorylation of proteins, both of which may stimulate intracellular protein synthesis (van Acker et al. 1999).

Gln is obtained by both absorption and for ingested nutrients and through biosynthesis. Gln from tissues maintains plasma concentrations between 0.6-0.9 mM, making it the most abundant free amino acid in plasma and intracellular pools (reviewed in (Smith 1990; Souba & Austgen 1990)). This is achieved by gln release from the carbon skeletons within muscle. The lungs also contain the prerequisite enzymatic machinery necessary for the *de novo* biosynthesis of gln that is quantitatively equal to muscle, while the brain and heart produce smaller amounts. Although these tissues generally provide an adequate supply, additional gln is occasionally required in the diet when body stores are insufficient, i.e. in stressed conditions such as injury and infection (Wu et al. 1991b). The importance of gln to suckling piglets is supported by the abundance of this amino acid in milk (1.9 and 3.4 mmol/L of defatted milk) on d 22 and 29 of lactation (Wu & Knabe 1994). However, the addition of gln to infant formulas is debated (Buchman 1996; Burke et al. 1989; Gianotti et al. 1995; Souba 1993; Zapata-Sirvent et al. 1994). Also, the the safety of pharmacological doses of gln is questioned (Buchman 1996) as it has been shown to increase hepatic aminotransferases (Hornsby-Lewis et al. 1994), exacerbate hepatic encephalopathy in patients with stable cirrhosis (Oppong et al. 1995) and may promote tumor growth (Chance et al. 1990). TPN solutions were traditionally free of gln because of its chemical instabilities under high temperature and its low solubility. However, these problems can be overcome by using gln dipeptides such as L-alanyl-1-gln and glycyl-1-gln (reviewed in (Shipley 1996)). Once gln is made available in the lumen of the intestine from the digestion of protein, sodium-dependent transport of gln across the apical and basal membranes occurs via several distinct transport mechanisms (i.e. systems A, ASC, N, L, and y+ (Su et al. 1997)), which also vary with ontogeny and between species (gln transport in human and rat compared in (Novak & Beveridge 1997)). However, little gln reaches the bloodstream because the absorptive cells of the small intestine utilize almost all that is absorbed from the lumen of the gut (Newsholme & Parry-Billings 1990; Wu 1998), in addition to extracting 20 to 30% of circulating gln from the bloodstream (Souba 1993).

Glutamine and Intestinal Function

Gln was demonstrated to be a key nutrient in the maintenance of the gut mucosa when addition of gln to TPN solutions (Alverdy 1990) or to the diet of newly weaned piglets (Wu et al. 1996) partially prevented intestinal atrophy. Gln has been shown to be beneficial in several models of bowel injury, but it has been difficult to differentiate whether these benefits are due to improvements in enterocyte function or to enhancement of gut immune function (Souba 1993). It has also been implicated in preserving intestinal metabolism, structure and function and was suspected to help accelerate healing of the gut mucosa in irradiated animals (via supporting mitosis in the proliferative zone of the villous crypts; (Klimberg et al. 1990)). Gln has also been shown to prevent or delay changes in gut permeability either by acting as a preferred fuel source and/or reestablishing gut microcirculation (Dugan & McBurney 1995).

Immunologic Influences of Glutamine

Upon encounter with antigen, immune cells seem to possess the ability to switch on transporter activity, potentially to increase the availability of amino acids during key periods such as activation or maturation (Crawford et al. 1994; Deves & Boyd 1998). The rate of gln utilization is hypothesized to remain high in order to provide optimal conditions for response to an immune challenge at any time (Newsholme & Parry-Billings 1990). Although specific gln transporters in immune cells have yet to be identified, changes in expression of these transporters would potentially be useful in regulating the availability of gln to immune cells, particularly during immune challenges (Newsholme & Parry-Billings 1990).

Gln exerts an influence on the immune system in several ways (reviewed in (Field et al. 2000a)). It is an extremely important fuel for both macrophages and lymphocytes and this may be the case for all cells of the immune system (Newsholme & Parry-Billings 1990). T cells are dependent on gln for optimal growth and function and feeding gln (six-fold higher than control subjects) by enteral tube feedings was shown to significantly increase the ratio of Th to Tc among experimental subjects (Jensen et al. 1996). Lymphocyte proliferation, B-cell differentiation, cytokine production (interleukin (IL)-1, IL-2, IFN-γ and TNF-α) and macrophage-mediated phagocytosis are all positively influenced by exogenous gln supply (Newsholme et al. 1985; Newsholme & Calder 1997; Parry-Billings et al. 1990; Ruggeberg et al. 1997; Tayeh & Marletta 1989; van Acker et al. 1999). Gln is also an important amino acid for sIgA synthesis (Alverdy 1990) and there is data to support the importance of gln for neutrophil bactericidal function (Ogle et al. 1994), and nitric oxide (NO) production (Murphy & Newsholme 1998; O'Dowd & Newsholme 1997).

The mechanism for the immunological influence of gln are not completely understood. The incomplete metabolism of gln to glutamate has been hypothesized as a way for cells to precisely regulate the production of biosynthetic precursors and to enable them to respond rapidly to immunological challenges (Newsholme et al. 1985). Gln also acts as an antioxidant by way of influencing glutathione levels, one of the most potent natural antioxidants produced by the gut and other tissues (Johnson et al. 2003). Gln starvation causes a rapid and profound decrease in glutathione levels (Johnson et al. 2003), which is involved in several fundamental biological functions, including free radical scavenging, detoxification or xenobiotics and carcinogens, redox reactions, and biosynthesis of DNA, proteins, and leukotrienes. Therefore, the augmentation of antioxidant protection provided by gln may decrease local immune activation, which prevents increased permeability caused by oxygen radicals produced by activated immune cells (van der Hulst et al. 1997).

Swine Glutamine Metabolism

Many studies investigating the cellular metabolism of gln have been performed in neonatal or young piglets, many of which were intended as models for neonatal humans. These studies have led to the discovery that piglets show developmental differences in metabolism of gln and related urea cycle intermediates. The early low citrulline synthesis (14-21 d) increases 10-20-fold that coincides with a 15-fold increase in the activity of P-5-C synthase (29-58 d) (Wu et al. 1994). Thus the low rate of citrulline synthesis from gln in enterocytes from suckling pigs provides a basis for explaining why arginine and proline are nutritionally essential amino acids for young suckling piglets but not for adult pigs (Wu et al. 1994). The influence of gln on immune function in infected pigs fed a gln-free diet, was demonstrated when the plasma concentration decreased (267 μmol/L vs 388 μmol/L in uninfected controls) and the proliferative response of lymphocytes to Con A stimulation were depressed (Yoo et al. 1997). In contrast, in all pigs that received dietary gln, intramuscular gln concentrations were significantly higher, suggesting improved gln status (Yoo et al. 1997).

1.8 Beta-glucans

Barley (1-3),(1-4)-B-D-glc is the major component of barley endosperm cells walls, accounting for about 75% (w/w) of total cell-wall carbohydrates, the remainder of which is protein and pentosans (Jiang & Vasanthan 2000). B-glc from barley consists of a linear chain of glucose residues, containing about 70% B(1-4) and 30% B(1-3) glycosidic links (described in (Jiang & Vasanthan 2000), shown in Figure 3).

Beta-Glucan Metabolism

In addition to their physiological effects associated with insoluble fiber, such as an increase in fecal bulk or the ability to relieve constipation, Barley B-glc also possess the biological benefits of soluble fiber, specifically influencing serum glucose and cholesterol levels (Izydorczyk et al. 2000). Wheat- and barley-derived B-glc (McIntosh et al. 1991) have been associated with reductions in serum cholesterol concentrations in both human and animals. More specifically, a decrease in low-density-lipoprotein cholesterol in hypercholesterolemic individuals was found following consumption of barley relative to wheat foods (McIntosh et al. 1991). Reductions in serum cholesterol levels are suggested to result from an increased fecal excretion of bile acids, modification of rates and site of absorption of nutrients in the gastrointestinal tract, effect from short chain fatty acids produced by fermentation in the colon, and alteration of insulin concentrations or sensitivity (Anderson & Siesel 1990).

Immunological Influences of B-glucans

For many years, B-glc were attributed with the anti-nutritional properties of grains such as barley and oats. However, more recently the focus of B-glc research has shifted from their anti-nutritional properties to their ability to influence immune function. This research began when the zymosan component responsible for macrophage activation was shown to be B-glc (Riggi & Di Luzio 1961). Since that time, B-glc from various plants, bacteria, and fungi have been shown to bind to receptors on macrophages and other white blood cells and activating them (Brown & Gordon 2001). Several receptors on macrophages, other leukocytes and non-immune cells that recognize B-glc have been described, including CR3, lactosylceramide, scavenger receptors, and Dectin-1 (Brown & Gordon 2003). A wealth of knowledge has emerged regarding the clinical use of B-glc and their obvious therapeutic potential (Willment et al. 2001) as with Lentinan, a B-glc from mushrooms, that has been used for human cancer therapy (reviewed in (Chihara 1992)). The most potent immunonutrients are the large molecular weight B-glc, which directly activate leukocytes *in vitro*, while low molecular weight B-glc activate leukocytes to a smaller extent and very short B-glc (<5000-10,000 molecular weight) are generally considered inactive (Brown & Gordon 2003).

Information on the mechanisms by which B-glc influence immune function is limited, but it has been suggested that B-glc may stimulate lymphocytes through a similar receptor to that which lectins bind (Willment et al. 2001), or similar to dietary fiber, may result from alterations in the gut microflora, production of SCFA and modulation of mucin production (reviewed in (Schley & Field 2002)). Proinflammatory responses induced by large molecular weight and particulate B-glc have been shown to involve mediators with a central role in signaling, including Toll-like receptors and Dectin-1, and downstream components such as MyD88 and NF-KB (Brown et al. 2003). The B-glc source may play an important role in the generation of these immune mediators, as even the B-glc from the fungi *Pneumocystis carinii* acts as a potent inducer of macrophage activation through NF-KB utilizing cellular receptors and signaling pathways distinct from lipopolysaccharide (LPS) (Lebron et al. 2003).

Beta-Glucan Supplementation in Swine

There are a few studies examining the effect of B-glc, both as an additive or as a constituent of major feed ingredients in swine diets (Decuypere et al. 1998; Dritz et al.

1995; Fortin et al. 2003; Hiss & Sauerwein 2003; Schoenherr et al. 1994). However, the effect of B-glc on the immune system is not established. Furthermore, the extent to which dietary B-glc come in direct contact with immune cells in GALT has not been examined.

Part III – Infection and Immune Response

1.9 Enterotoxigenic *Eschericia coli*

Esecherichia coli infection and clinical diarrhea

The clinical manifestation of *E. coli* infection is known as infectious diarrhea (reviewed in (Casburn-Jones & Farthing 2004)). It is the most common cause of diarrhea worldwide, is the leading cause of death in childhood and the second most common cause of death worldwide. Diarrheal disease is estimated to kill 4 to 6 million children globally each year (Levine & Edelman 1984). In the United States alone, the incidence of acute diarrhea in children less than 3 y old is on the order of 1.3-2.3 episodes per child per year, adding to a combined cost of greater than 2 billion per year for inpatient and outpatient care for pediatric diarrhea (Glass et al. 2001). Currently, there are four recognized classes of *E. coli* that cause diarrheal disease in humans; enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli*, enteroinvasive *E. coli* and enterohemorrhagic *E. coli* (Levine & Edelman 1984). Each of these types of *E. coli* manifest distinct pathogenesis, clinical syndrome, and epidemiology. ETEC is identified as a causative agent in approximately 800,000 cases in children under 5 years of age, in countries such as Nicaragua, Chile, Bangladesh and Mexico (Black et al. 1981; Cravioto et al. 1988; Levine & Noriega 1993; Paniagua et al. 1997). ETEC is also responsible for diarrhea in travelers from industrialized areas that visit less developed countries (Levine & Edelman 1984).

Although oral rehydration therapy remains central to managing cases of diarrhea, more recent therapies include antimotility agents, antisecretory agents, antimicrobial therapy and probiotics (Casburn-Jones & Farthing 2004). There has been considerable work on the composition of the oral rehydration therapies including the addition of glucose plus amino acids aimed at stimulating fluid absorption across separate Na+-coupled carriers in the apical membrane (Patra et al. 1989; Rombeau 1990).

ETEC diarrhea is caused by a choleragen-like enterotoxin (LT) and/or a heat-stable enterotoxin (ST) released by E.coli after it adheres to an intestinal epithelial cell (reviewed in (Gyles 1992)). While heat-stable enterotoxin causes an increase in the cGMP levels in intestinal cells, heat-labile enterotoxin stimulates the production of cAMP by the enterocytes (Field 1976; Moon 1978). These enterotoxins induce intestinal secretion by similar mechanisms. Overproduction of cAMP stimulates both the secretion of electrolytes by the cells of the crypt and inhibits the absorption of electrolytes by the cells of the villi, there is a rapid increase in the rate of secretion of electrolytes and water from the intestine, leading to diarrhea, severe dehydration and sometimes death (Holmgren 1981). In the presence of normal colonic transport function, diarrhea ensues only when the volume originating from the small intestine exceeds the large reserve capacity of the colon.

Swine ETEC Infections

Infections with ETEC are also a major concern to several livestock species, including cattle (Contrepois et al. 1998) and swine (Nabuurs 1998). ETEC induce diarrhea in swine during the neonatal period (<6 days of age), and immediately after weaning (3 to 9 weeks old) (Dean et al. 1989). Therefore, reducing the risk of ETEC infection in piglets would be both beneficial to swine producers and have public health benefits. In contrast to the situation with human infants, there are few options for the treatment of diarrhea in young piglets. Therefore, dietary intervention aimed at improving the development of the immune system and the intestine would be an alternative method of helping reduce the risk of infection in piglets.

Since piglets are born free of immunoglobulins and acquire them quickly from colostrum, the intestinal epithelium must develop a mechanism for taking up physiologically active macromolecules (Lecce 1975). The neonatal gut does not appear to be able to clearly and consistently distinguish useful macromolecules from potentially harmful macromolecules. Possibly, this non-discriminating absorption that occurs in the neonate and not in the adult, could account for some of the vulnerability of the neonatal animal to intestinal microorganisms. In addition to their reliance on maternal immunity, intensive agriculture practices have resulted in piglets, formerly weaned at ten to twelve weeks of age, being weaned at week two to five (Nabuurs 1998). Weaning not only suddenly severs piglets from the source of antibodies and protective factors, as well as easily digestible and absorbed nutrients in the milk of the dam, piglets are more commonly weaned at a time when the immune system has not had sufficient time to mature. In addition, newly weaned piglets have a shortage of functional and/or appropriate digestive enzymes, exposure to environmental stress, increased gastric pH, differences in expression of cell-surface molecules, villus atrophy and a novel feeding regimen along with a decreased feed intake (FI) (Nabuurs 1998; Sarmiento et al. 1988). The combination of early weaning to plant proteins and a sudden halt in the supply of maternal antibodies results in adverse reactions of the piglet and diarrhea is the first and most prevalent clinical sign of illness after early weaning (Nabuurs 1998).

K88 Antigens

Infection with ETEC is most commonly confirmed by identification of the specific fimbrial adhesins present on the surface of the isolated bacterium (Thorns et al. 1989). The pathogenesis of diarrhea involves adherence to the small intestine by means of filamentous surface appendages called pili or fimbriae, followed by hypersecretion of water and electrolytes caused by bacterial enterotoxins (heat stable enterotoxin: STa, and heat labile enterotoxin: STb). Antigenically different types of pili described for ETEC of swine origin include: K88 (F4), K99 (F5), 987P (F6), and F41 (Fairbrother et al. 1986; Nagy et al. 1992; Sarmiento et al. 1988; Thorns et al. 1989). Susceptibility of piglets to pilus-mediated adherence is age specific. While neonatal piglets are susceptible to strains of ETEC that produce 987P or K99 pili, whereas ETEC that produce K88 pili are commonly associated with diarrheal disease in both neonatal and newly weaned piglets (Sellwood 1979). Data from the U.S. indicate that more than one half of the ETEC strains in both age groups are K88$^+$ (Nagy et al. 1992). Bacteria which express the K88 antigen possess a number of unique characteristics, such as showing B-haemolysis when cultured on blood agar (Sellwood 1979). Adhesion is also mannose resistant, inhibited by the presence of specific antiserum, and requires pilus expression (growth of the organisms in temperatures greater than 18°C) (Laux et al. 1986). The K88 antigen contains two plasmids, one encoding the genes for K88 fimbriae on the bacterial surface, and a second plasmid encoding the genes for toxin production (Blomberg et al. 1993). The K88 antigen is contained in a fimbrial structure which is commonly known as the K88 adhesin, and exists in at least three different antigenic forms (K88ab, K88ac, and K88ad) which appear to have different specificities for brush border membranes isolated from pigs of differing phenotype (Laux et al. 1986).

Cell Surface Characteristics

Adhesion of ETEC not only involves expression of fimbriae, but also the cell surface characteristics of the host. Pilus-specific binding sites must be available on intestinal epithelium for pilus-mediated adherence (Dean et al. 1989), which is an essential component for the successful host colonization and enterotoxin delivery to the host. The affinities of these receptor-ligand interactions as well as the concentrations of the interacting molecules and the presence of nutritional and inhibitory components will determine the level of success of the bacterial colonization process. This adhesion is mostly mediated by bacterial fimbrial-determined receptor specificity and therefore tropism exists for particular host species, ages, intraspecies variants, and intestinal segments (Khan et al. 1996). ETEC fimbriae are generally believed to recognize the carbohydrate moieties of host glycoconjugates. Binding sites for K88 antigens have been characterized. While the K88ad adhesin appears to preferentially bind to glycolipids, the K88ab and K88ac adhesins preferentially bind to glycoproteins (reviewed in (Jin & Zhao

2000)). Among the binding sites for K88ac are intestinal mucin-type glycoprotein (IMTGP) 1 and IMTGP 2 (Grange et al. 1998). Proteins of various sizes in both intestinal mucus and brush border membranes were recognized by K88ab fimbriae, including a GP74 mucosal transferring (Grange & Mouricout 1996). An intestinal neutral glycosphingolipid was identified as a phenotype-specific receptor for the K88ad fimbrial adhesion (Grange et al. 1999).

K88+ bacteria do not adhere to the brush borders of all pigs as susceptibility to epithelial cell colonization is mediated by the presence of binding sites for the K88 antigen, a character conferred by an autosomal dominant gene (Sarmiento et al. 1988). Both homozygous dominants and heterozygotes possess the receptor and are thus susceptible to infection, while homozygous recessive piglets are not because it is absent (Sellwood 1979).

In order to bind to the underlying epithelial cells, K88-bearing *E.coli* strains must pass through the mucus layer. Receptors specific for the K88ab adhesins are present in both the soluble crude mucus preparations and the brush border preparations, but whether the mucus layer serves as an initial point of contact for ETEC adhesion or the soluble receptors serve to prevent colonization by interfering with the attachment ETEC to the underlying epithelial cells is unclear (Laux et al. 1986). As compared to 5-week-old piglets, a relatively small amount of K88-specific receptor (1/16th) is present in newborn piglet ileal mucus, suggesting that the mucus layer plays a protective role (Conway et al. 1990). A relatively large amount of K88-specific receptor was found in 35-day-old piglet ileal mucus, which was concentrated enough to bind to E.coli K88-fimbriated cells and prevent the strain from binding to ileal epithelial cells supports this theory (Conway et al. 1990). Although the amount of K88 receptor present in newborn ileal mucus is insufficient to prevent adhesion, older animals appear to develop the ability to synthesize sufficient K88 receptor in their mucus to ensure protection.

E. coli infection models

The pathogenesis of *E.coli*-induced postweaning diarrhea in swine is not well defined. Despite efforts to design a completely reproducible ETEC model to properly study the various stages of the infection, few researcher have been successful (reviwed in (Sarmiento et al. 1988).

1.10 Immune Response to E. coli Infection

The Epithelial Barrier

Epithelial cells are responsible not only for absorbing nutrients from the lumen, but also for barricading the hostile external environment form the internal milieu. Challenges to GALT by pathogens (i.e. ETEC) stimulate a rapid response in the piglet. This process involves the nonimmunologic components of mucosal barrier function such as vomiting, increased propulsion, hypersecretion of electrolytes, water and mucus, increased blood flow and epithelial restitution (reviewed in (Holtug et al. 1996)).

Innate Immune Response to Infection

The innate immune response is the first line of defense against microbial pathogens. A wide variety of bacterial components (i.e. peptidoglycan, lipoteichoic acid, lipoarabinomannan, lipopeptides, bacterial DNA, and LPS) are capable of stimulating the innate immune system, whose principal challenges are pathogen recognition, mounting an immediate defense response, and activating adaptive immune responses (desribed in (Miyake 2003)). LPS is a principal component of Gram-negative bacteria that potently activates the innate immune system and is one of the best studied microbial products (Ulevitch & Tobias 1995). The intravenous administration of LPS has been reported to produce diarrhea and intestinal lesions in a variety of animal species (Mittermayer et al. 1982) and also causes glucose uptake in rats to support the numerous immunological functions of LPS-stimulated macrophages and lymphocytes (Lang et al. 1991). LPS is also known to increase the synthesis and/or release of cytokines and lipid mediators in the gut (TNF-α, IFN-γ, IL-1, IL-6, platelet-activating factor, and arachidonic acid metabolites) (Fong et al. 1990; Peskar et al. 1981; Sun & Hsueh 1988; Touchette et al. 2002; Webel et al. 1997). Despite its potent immunostimulatory activity, the mechanisms underlying LPS recognition have not been clearly defined and only recently have molecules constituting the LPS recognition compex (CD14, TLR4, and MD-2) been identified (Miyake 2003).

Neutrophils occupy a major role in the innate immune response to ETEC and are recruited abundantly to sites of infection (reviewed in (Sandborg & Smolen 1988). This recruitment is the result of complex signaling events between PMN and epithelial cells that are triggered by the attachment of bacterial pathogens to the epithelial surface. Binding of bacteria results in the secretion of potent chemoattractants by epithelial cells, such as IL8 from the basolateral surface (Colgan et al. 1996; Eckmann et al. 1993; McCormick et al. 1993) and a pathogen-elicited-epithelial-chemoattractant (McCormick et al. 1998). This apically secreted pathogen-elicited-epithelial-chemoattractant was further characterized as the eicosanoid hepoxillin A_3 (Mrsny et al. 2004) and directs PMN migration across an epithelial monolayer through tight junctions. After entering infected tissue, neutrophils non-specifically engulf the invading bacteria (by phagocytosis (Hofman et al. 2000)), become activated and secrete a number of inflammatory agents such as cytokines and reactive oxygen intermediates (ROI: O2-, H2O2, and other reactive O2 metabolites (Baker & Campbell 1991; Mobert et al. 1999) aimed at destroying the bacteria (Hofman et al. 2000; Sandborg & Smolen 1988).

Adaptive Immune Response to Infection

In order to mount an adaptive immune response, antigens must be transported across the gut to the underlying tissues by way of epithelial cells or by M cells, which act as APC and allow antigen to come in contact with dendritic APC and classical phagocytic macrophages. M cells (first described in (Owen 1977; Owen & Jones 1974)) form a latticework with many IELs (Laissue et al. 1993). There are also many lymphocyte-containing cytoplasmic pockets at the basolateral surface of M cells (Jepson & Clark 1998). Therefore, the structure of M cells makes them an ideal gateway for the presentation of enteric antigens to the cells of the immune system. Certain microorganisms are known to exploit M cells as a route of host invasion (a description of these pathogens has been reviewed elsewhere (Gebert et al. 1996; Savidge 1996; Siebers & Finlay 1996). Once these antigens have been delivered to the underlying lymphoid tissue, an immune response is activated.

The humoral response is initiated by antigen uptake by APCs, degradation into immunogenic peptides, and presentation on the cell surface within the context of MHC class II molecules. Specific T cells recognize and bind to this complex, and subsequently release various cytokines (IL-4, IL-5, IL-6) to activate B cells, other T cells and macrophages. Once activated, precursor B cells undergo immunoglobulin class switching from IgM to IgA (not IgG) and amplification with enhanced IgA antibody production specific for the antigen sampled from the intestinal lumen. Migration of antigen-specific B- and T-cells from GALT to blood occus within 5-7 d following antigen exposure (Kantele et al. 1996; Quiding-Jarbrink et al. 1995) eventually homing back and becoming established in GALT (Rothkotter et al. 1999). The principal Ig of mucosal defense is secreted IgA (Insoft et al. 1996), and sIgM to a lesser extent (Brandtzaeg et al. 1987). Plasma cells continue to actively produce antigen-specific antibody for a variable period as plasma cells (of mice) have been reported with a half-life from 4.7 days up to 8 weeks (Mattioli & Tomasi, Jr. 1973) and it was reported that intestinal humoral immunity may be dependent on plasma cells that have a life span of more than 1 year (Slifka et al. 1998). Secreted Ig bind to and coat the surface of potential pathogens, thus preventing their attachment to the glycocalyx of the surface epithelium (Alverdy 1990; Keren 1992).

The cellular immune response stimulates T cells to develop into effector cells (Tc, CD8+), which recognize MHC I-antigen complexes and secrete bioactive factors that result in the direct killing of the infected cell (reviewed in (Gaskins 1998). Degraded intracellular pathogens are presented in the context of self-MHC class I antigens and similar to the humoral clonal expansion of B cells, Tc recognize a single antigen and clonally expand in numbers. In GALT, activated T cells leave the PP, eventually returning to the epithelium (IELs)(Brandtzaeg et al. 1989) or the lamina propria (Agace et al. 2000; Langkamp-Henken et al. 1992). The processes of cell adhesion, migration and signaling (reviewed in (Butcher et al. 1999; Gonzalez-Amaro & Sanchez-Madrid 1999)) are mediated by specific cell-surface molecules; selectins (Yang & Binns 1993a), integrins (Butcher 1991; Holzmann & Weissman 1989) and CD44 and their ligands (Yang & Binns 1993b).

1.11 Conclusions

The gastrointestinal tract is a unique organ system because not only is it responsible for absorption of a very large volume of fluid and nutrients, but it is also the largest immune organ in the body. Many immune cell populations reside in GALT, which can be futher divided into nonaggregated and aggregated tissues. Although the swine immune system is similar in several ways with other species, they have been shown to have a very diverse T lymphocyte population, differences in immune cell proportions, and specific PP structures. Many changes occur in the swine immune system as the animal matures. Although many of the changes that occur during development have been characterized, the functional consequences are poorly understood.

The importance of nutrition to the immune system is well established. Nutrition is especially important in young piglets that first rely on maternal colostrum and milk not only to meet their nutrient requirements, but to also obtain passive immunity. Piglets are then rapidly weaned onto plant-based diets and the weaning process is associated with adverse reactions (i.e. growth lag, infections) that are more frequent with early weaning. The nutrient requirements of swine have been defined and are optimized for growth, a process that has been facilitated with the development of new analytical techniques such as the indicator amino acid method. However, the ideal proportions of nutrients for young piglets and human infants that would maximize immune function are less well defined. While producers and health care providers rely on antibiotics to treat infection and promote growth, there is a growing movement to minimize antibiotic usage. As a result, attention has shifted to naturally-occuring molecules. Two potential nutrients with immunological properties are the amino acid gln and Barley-derived B-glc. There is considerable literature to support the importance of both gln and B-glc to the immune system. Most of this literature originates from studies performed in other species (i.e. human or rodent) and little is established in the young piglet. Furthermore, knowledge of the mechanisms by which specific nutrients influence immune function are limited and immune function is rarely studied in GALT, the logical site for a diet-immune interaction.

Newly weaned piglets and neonatal babies are susceptible to gastrointestinal infections, such as those caused by ETEC. Several strains of ETEC have been identified that cause disease in these species. Unfortunately, determination of the mechanisms of infection and design of potential intervention strategies are limited. This is because of the lack of reproducible, experimental model of ETEC infection. Despite advances in the understanding of the systemic immune response to infection, there are gaps in the literature on the response of GALT tissue.

Table 1. Cluster of Differentiation Antigens and their function

CD antigen	**Expression**	**Function**
CD2	T cells, NK cells	Low affinity, non-conventional adhesion molecule, binds LFA-3 in humans and CD48 in rats, interactions in the T cell-APC contact zone facilitates scanning of MHC-peptide complexes by TCR and significantly enhances TCR engagement (van der Merwe & Davis 2003)
CD3	T cells	Component of TCR complex, CD3 consists of the γ, δ, ε and ζ chains (Weiss 1993); cytoplasmic domains involved in coupling between the antigen specific signal delivered by the T cell receptor and the intracellular activation pathways (Pescovitz et al. 1998); Several components of the CD3 complex are essential in pre-TCR assembly and function during T cell development in the thymus (Haks et al. 1999)
CD4	Th	Monomeric polypeptide, TCR co-receptor, binds to MHC class II molecules (van der Merwe & Davis 2003)
CD8	Tc	Disulphide-linked dimmer, TCR co-receptor, binds with low affinity to MHC class I molecules (van der Merwe & Davis 2003)
CD18/ CD11	PMNs, leukocytes	significant in PMN migration across both epithelia and endothelia (Jaye & Parkos 2000); heterodimeric integral membrane glycoproteins expressed solely on leukocytes; consist of a common B chain (CD18) that associates with one of four alpha chains (CD11a, CD11b, CD11c, CD11d) (Springer 1995)
CD25	T cells	IL-2 receptor comprised of three subunits; α, functions in IL-2 binding, β & γ – function to augment ligand binding and induce cellular signaling (Malek 2003)
CD44	leukocytes, erythrocytes, fibroblasts, and epithelial cells	Family of general adhesion surface molecules with a wide tissue distribution, anti-CD44 Abs stain most of human and porcine PBL, involved in broad range of lymphocyte activities, such as lymphohemopoiesis, cell-cell adhesion, cell-matrix interaction, lymphocyte migration, and lymphocyte activation (Yang & Binns 1993b)
CD45	T cells	Component of multimeric T cell receptor complex (Woodward et al. 1995); Several isoforms in swine (RAC, RA, RC, RO) (Bailey et al. 1998; Haverson et al. 1999); RA identifies quiescent naïve-type T cells and high proportion in young animals, RO expressed on antigen mature cells (Field et al. 2000b)
CDw75	Mature B cells, red blood cells, epithelial cells	Functions in B-B cell adhesion; ligand for CD22, expressed on germinal centre B cells (Tanimoto & Ohtsuki 1996)

Table 2. Cytokines and their functions and sources

Cytokine	Sources	Function
IL-2	Recently activated T cells	Th1-type cytokine; Most critical cytokine for promoting clonal expansion of recently activated T cells, induce biological response after binding to high affinity receptor (CD25) (Malek 2003)
IL-4	T cells	Th2-type cytokine which stimulates B cell proliferation and differentiation, influences intercellular associations and permeability across epithelial and endothelial cells
IL-10		Th2-type cytokine with ability to down-regulate Th1 responses (IFNγ secretion and activation of monocytes/macrophages), important in Th2-mediated inflammatory processes (Groux & Cottrez 2004)
TNF-α	Monocytes, fibroblasts, and endothelial cells	Th1-type- and proinflammatory cytokine; bind to cell surface receptors, leading to the rapid production and secretion of IFN-gamma, serves as a first line of defense against invading pathogens
IFN-γ	NK, CD8 & CD4+ T cells, macrophages, dendritic cells, B cells	Th1-type pleotropic cytokine that plays an essential role in both the innate and adaptive phases of an immune response (Szabo et al. 2003); induces increased intestinal epithelium permeability (Heyman & Desjeux 2000; Madara & Stafford 1989)

Table 3. Amino acids categorized by degree of indispensability and studies examining their requirements in piglets.

Dispensable	**Conditionally Indispensable**	**Indispensible**
Alanine	Gln (Robinson et al. 1999)	Lysine (House et al. 1998)
Aspartate	Arginine (MacDonald et al. 1984)	Isoleucine (Elango et al. 2002)
Asparagine	Cysteine (Vina et al. 1995)	Leucine (Elango et al. 2002)
Glutamate	Glycine (Ngo et al. 1977)	Valine (Elango et al. 2002)
Serine	Proline (Brunton et al. 1999)	Methionine (Shoveller et al. 2003)
	Tyrosine (Bross et al. 2000)	Phenylalanine (House et al. 1997)
		Threonine (Bertolo et al. 1998)
		Tryptophan (Zimmerman 1975)
		Histidine (Zhang et al. 2002)

Table 4. Transport properties of ions relevant to intestinal transport

Ions	**Luminal Surface (outward facing)**	**Basolateral Surface (inward facing)**
Sodium (Na$^+$)	Permeable to Na$^+$ transport because of low intracellular Na+ maintained by active extrusion of Na+ across the basolateral membrane, steep electrochemical gradient partly due to negative potential difference across this membrane, Only ion that moves from a lower to a higher electrochemical gradient (Ussing & Zerahn 1951)	Impermeable to Na$^+$ transport; active tranpsort mechanism (Na-K-ATPase) pumps Na$^+$ to the inside solution coupled with K$^+$ transport in the opposite direction (ratio of 3:2) (Ussing 1994), maintains high K+ and low Na+ concentration in the cell; absorption of nutrients critically dependent on Na+K+ATPase, which maintains a low intracellular Na+ concentration (Koefoed-Johnsen & Ussing 1958).
Potassium (K$^+$)	Impermeable to K$^+$ transport	Na+K+ATPase pumps K$^+$ to the outside solution coupled with Na$^+$ transport, K+ ions free to return to the internal solution because of the high K+ permeability of the basolateral membrane (Ussing 1994)
Chloride (Cl$^-$)	Passes through specific Cl$^-$ channels which do not admit H_2O (Ussing 1994), secretion occurs in both the villar and crypt area (Holtug et al. 1996)	driving force for luminal secretion maintained by activation of basolateral Na+K+2Cl- cotransporter – increases the intracellular level of Cl- above its electrochemical equilibrium, exit of K+ through basolateral channels maintains intracellular electronegativity necessary for Cl- secretion (Holtug et al. 1996)
Carbonate (HCO_3^-)	Involved in electrolyte secretion by its serosal-to-luminal transcellular transport	
Water (H_2O)	H_2O follows electrogenic Na+ transport during absorption, follows Cl- during electrogenic secretagogue-induced secretion (Holtug et al. 1996), large proportion utilizes paracellular pathways or specific H_2O channels (Ussing 1994)	

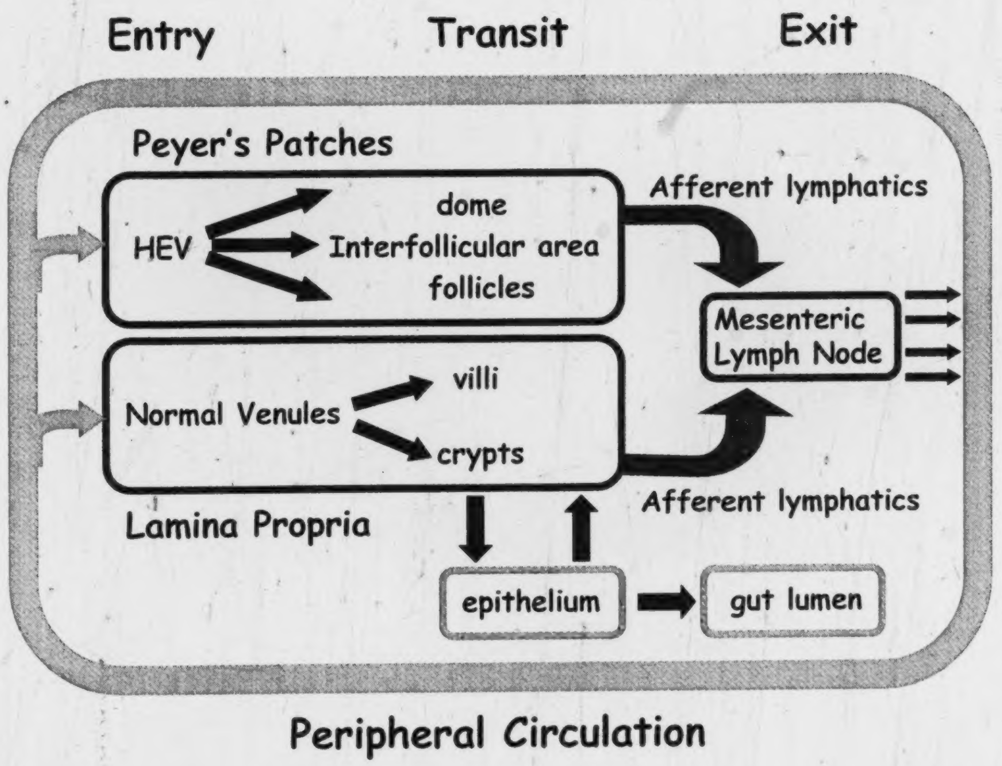


Figure 1 – Patterns of Lymphocyte Trafficking (adapted from (Rothkotter et al. 1999)). From the peripheral circulation, lymphocytes enter PP via high endothelial venules (HEV) and enter lamina propria via mucosal blood vessels. Following antigen exposure at these sites, lymphocytes and dendritic cells filter to the mesenteric lymph nodes via afferent lymph vessels. Unlike other species, piglet T cells leave the mesenteric lymph node to the peripheral circulation via an unusual route of migration; directly via blood capillaries rather than in efferent lymph.

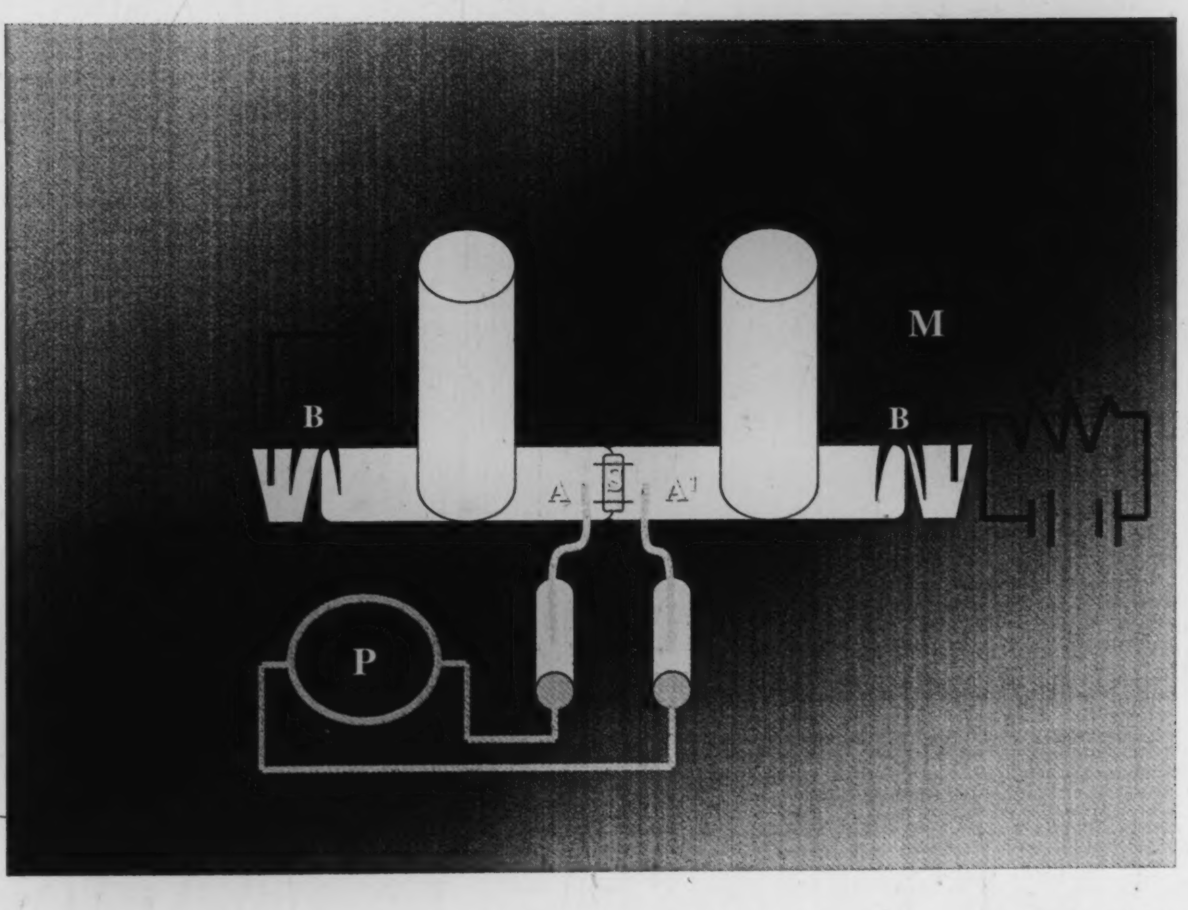


Figure 2. Ussing Chamber Diagram (adapted from (Ussing & Zerahn 1951)). One pair of electrodes, preferably connected via agar bridges (A & A^1), is placed adjacent to the tissue (S) for measurements of spontaneous Potential Difference (PD – read on tube potentiometer). Short-cicuit current (Isc) passing tissue read on Microamperemeter (M) by means of current passing electrodes (B) are placed in the periphery of the chambers.

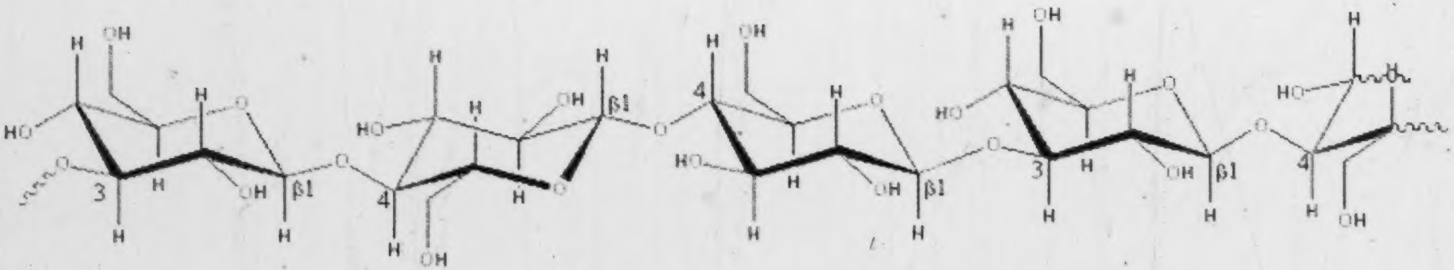


Figure 3 – Molecular B-glc structure

1.12 Refences

Agace,W.W., Higgins,J.M., Sadasivan,B., Brenner,M.B., and Parker,C.M. 2000. T-lymphocyte-epithelial-cell interactions: integrin alpha(E)(CD103)beta(7), LEEP-CAM and chemokines. Curr. Opin. Cell Biol. 12: 563-568.

Albin,D.M. and Tappenden,K.A. 2001. Advances in methods to evaluate gastrointestinal transport function. Curr. Opin. Clin. Nutr. Metab Care 4: 351-354.

Alverdy,J.C. 1990. Effects of Glutamine-Supplemented Diets on Immunology of the Gut. Journal of Parenteral and Enteral Nutrition 14: 109S-113S.

Andersen,J.K., Takamatsu,H., Oura,C.A., Brookes,S.M., Pullen,L., and Parkhouse,R.E. 1999. Systematic characterization of porcine ileal Peyer's patch, I. apoptosis-sensitive immature B cells are the predominant cell type. Immunology 98: 612-621.

Anderson,J.W. and Siesel,A.E. 1990. Hypocholesterolemic effects of oat products. Adv. Exp. Med. Biol. 270: 17-36.

Ardawi,M.S. and Newsholme,E.A. 1983. Glutamine metabolism in lymphocytes of the rat. Biochem. J. 212: 835-842.

Ardawi,M.S. and Newsholme,E.A. 1985. Fuel utilization in colonocytes of the rat. Biochem. J. 231: 713-719.

Bailey,M., Miller,B.G., Telemo,E., Stokes,C.R., and Bourne,F.J. 1993. Specific immunological unresponsiveness following active primary responses to proteins in the weaning diet of piglets. Int. Arch. Allergy Immunol. 101: 266-271.

Bailey,M., Plunkett,F., Clarke,A., Sturgess,D., Haverson,K., and Stokes,C. 1998. Activation of T cells from the intestinal lamina propria of the pig. Scand. J. Immunol. 48: 177-182.

Baker,S.S. and Campbell,C.L. 1991. Rat enterocyte injury by oxygen-dependent processes. Gastroenterology 101: 716-720.

Barman,N.N., Bianchi,A.T., Zwart,R.J., Pabst,R., and Rothkotter,H.J. 1997. Jejunal and ileal Peyer's patches in pigs differ in their postnatal development. Anat. Embryol. (Berl) 195: 41-50.

Barrett,K.E. 1993. Positive and negative regulation of chloride secretion in T84 cells. Am. J. Physiol 265: C859-C868.

Becker,B.A. and Misfeldt,M.L. 1993. Evaluation of the mitogen-induced proliferation and cell surface differentiation antigens of lymphocytes from pigs 1 to 30 days of age. J. Anim Sci. 71: 2073-2078.

Bennell,M.A. and Husband,A.J. 1981. Route of lymphocyte migration in pigs. II. Migration to the intestinal lamina propria of antigen-specific cells generated in response to intestinal immunization in the pig. Immunology 42: 475-479.

Bertolo,R.F., Chen,C.Z., Law,G., Pencharz,P.B., and Ball,R.O. 1998. Threonine requirement of neonatal piglets receiving total parenteral nutrition is considerably lower than that of piglets receiving an identical diet intragastrically. J. Nutr. 128: 1752-1759.

Bianchi,A.T., Zwart,R.J., Jeurissen,S.H., and Moonen-Leusen,H.W. 1992. Development of the B- and T-cell compartments in porcine lymphoid organs from birth to adult life: an immunohistological approach. Vet. Immunol. Immunopathol. 33: 201-221.

Binns,R.M. 1982. Organisation of the lymphoreticular system and lymphocyte markers in the pig. Vet. Immunol. Immunopathol. 3: 95-146.

Bjorksten,B. 1999. Environment and infant immunity. Proc. Nutr. Soc. 58: 729-732.

Black,R.E., Merson,M.H., Huq,I., Alim,A.R., and Yunus,M. 1981. Incidence and severity of rotavirus and Escherichia coli diarrhoea in rural Bangladesh. Implications for vaccine development. Lancet 1: 141-143.

Blecha,F., Pollmann,D.S., and Nichols,D.A. 1983. Weaning pigs at an early age decreases cellular immunity. J. Anim Sci. 56: 396-400.

Blomberg,L., Henriksson,A., and Conway,P.L. 1993. Inhibition of adhesion of Escherichia coli K88 to piglet ileal mucus by Lactobacillus spp. Appl. Environ. Microbiol. 59: 34-39.

Bode,B.P. and Souba,W.W. 1999. Glutamine transport and human hepatocellular transformation. JPEN J Parenter. Enteral Nutr 23: S33-S37.

Brand,K., Fekl,W., von Hintzenstern,J., Langer,K., Luppa,P., and Schoerner,C. 1989. Metabolism of glutamine in lymphocytes. Metabolism 38: 29-33.

Brandtzaeg,P., Bjerke,K., Kett,K., Kvale,D., Rognum,T.O., Scott,H., Sollid,L.M., and Valnes,K. 1987. Production and secretion of immunoglobulins in the gastrointestinal tract. Ann. Allergy 59: 21-39.

Brandtzaeg,P., Halstensen,T.S., Kett,K., Krajci,P., Kvale,D., Rognum,T.O., Scott,H., and Sollid,L.M. 1989. Immunobiology and immunopathology of human gut mucosa: humoral immunity and intraepithelial lymphocytes. Gastroenterology 97: 1562-1584.

Bross,R., Ball,R.O., Clarke,J.T., and Pencharz,P.B. 2000. Tyrosine requirements in children with classical PKU determined by indicator amino acid oxidation. Am. J. Physiol Endocrinol. Metab 278: E195-E201.

Brown,D.R. and O'Grady,S.M. 1997. Regulation of ion transport in the porcine intestinal tract by enteric neurotransmitters and hormones. Comp. Biochem. Phys., Part A. Phys. 118: 309-317.

Brown,G.D. and Gordon,S. 2001. Immune recognition. A new receptor for beta-glucans. Nature 413: 36-37.

Brown,G.D. and Gordon,S. 2003. Fungal beta-glucans and mammalian immunity. Immunity. 19: 311-315.

Brown,G.D., Herre,J., Williams,D.L., Willment,J.A., Marshall,A.S., and Gordon,S. 2003. Dectin-1 mediates the biological effects of beta-glucans. J. Exp. Med. 197: 1119-1124.

Brown,P.J. and Bourne,F.J. 1976. Development of immunoglobulin-containing cell populations in intestine, spleen, and mesenteric lymph node of the young pig, as demonstrated by peroxidase-conjugated antiserums. Am. J. Vet. Res. 37: 1309-1314.

Brunton,J.A., Bertolo,R.F., Pencharz,P.B., and Ball,R.O. 1999. Proline ameliorates arginine deficiency during enteral but not parenteral feeding in neonatal piglets. Am. J. Physiol 277: E223-E231.

Buchman,A.L. 1996. Glutamine: Is It a Conditionally Required Nutrient for the Human Gastrointestinal System? Journal of the American College of Nutrition 15: 199-205.

Burke,D.J., Alverdy,J.C., Aoys,E., and Moss,G.S. 1989. Glutamine-supplemented total parenteral nutrition improves gut immune function. Arch. Surg. 124: 1396-1399.

Butcher,E.C. 1991. Leukocyte-endothelial cell recognition: three (or more) steps to specificity and diversity. Cell 67: 1033-1036.

Butcher,E.C., Williams,M., Youngman,K., Rott,L., and Briskin,M. 1999. Lymphocyte trafficking and regional immunity. Adv. Immunol. 72: 209-253.

Casburn-Jones,A.C. and Farthing,M.J. 2004. Management of infectious diarrhoea. Gut 53: 296-305.

Chance,W.T., Cao,L.Q., and Fischer,J.E. 1990. Response of tumor and host to hyperalimentation and antiglutamine treatments. JPEN J. Parenter. Enteral Nutr. 14: 122-128.

Chihara,G. 1992. Recent progress in immunopharmacology and therapeutic effects of polysaccharides. Dev. Biol. Stand. 77: 191-197.

Chu,R.M., Glock,R.D., Ross,R.F., and Cox,D.F. 1979. Lymphoid tissues of the small intestine of swine from birth to one month of age. Am. J. Vet. Res. 40: 1713-1719.

Colgan,S.P., Dzus,A.L., and Parkos,C.A. 1996. Epithelial exposure to hypoxia modulates neutrophil transepithelial migration. J. Exp. Med. 184: 1003-1015.

Contrepois,M., Bertin,Y., Pohl,P., Picard,B., and Girardeau,J.P. 1998. A study of relationships among F17 a producing enterotoxigenic and non-enterotoxigenic Escherichia coli strains isolated from diarrheic calves. Vet. Microbiol. 64: 75-81.

Conway,P.L., Welin,A., and Cohen,P.S. 1990. Presence of K88-specific receptors in porcine ileal mucus is age dependent. Infect. Immun. 58: 3178-3182.

Cornes,J.S. 1965. Peyer's patches in the human gut. Proc. R. Soc. Med. 58: 716.

Cravioto,A., Reyes,R.E., Ortega,R., Fernandez,G., Hernandez,R., and Lopez,D. 1988. Prospective study of diarrhoeal disease in a cohort of rural Mexican children: incidence and isolated pathogens during the first two years of life. Epidemiol. Infect. 101: 123-134.

Crawford,D.H., Chen,S., and Boyd,C.A. 1994. Cationic amino acid transport in human T lymphocytes is markedly increased in the CD45RA, CD8+ population after activation. Immunology 82: 357-360.

Crockett,M. 1995. Physiology of the neonatal immune system. J. Obstet. Gynecol. Neonatal Nurs. 24: 627-634.

Cromwell,G.L. 2002. Why and how antibiotics are used in swine production. Animal Biotech. 13: 7-27.

Dean,E.A., Whipp,S.C., and Moon,H.W. 1989. Age-specific colonization of porcine intestinal epithelium by 987P-piliated enterotoxigenic Escherichia coli. Infect. Immun. 57: 82-87.

Decuypere,N., Dierick,N., and Boddez,S. The potentials of immunostimulatory substances (á-1,3/1,6 glucans) in pig nutrition. J.Anim.Feed.Sci. 7, 259. 1998.

Deves,R. and Boyd,C.A. 1998. Transporters for cationic amino acids in animal cells: discovery, structure, and function. Physiol Rev. 78: 487-545.

Dritz,S.S., Shi,J., Kielian,T.L., Goodband,R.D., Nelssen,J.L., Tokach,M.D., Chengappa,M.M., Smith,J.E., and Blecha,F. 1995. Influence of dietary beta-glucan on growth performance, nonspecific immunity, and resistance to Streptococcus suis infection in weanling pigs. J. Anim Sci. 73: 3341-3350.

Dugan,M.E. and McBurney,M.I. 1995. Luminal glutamine perfusion alters endotoxin-related changes in ileal permeability of the piglet. JPEN J. Parenter. Enteral Nutr. 19: 83-87.

Eckmann,L., Kagnoff,M.F., and Fierer,J. 1993. Epithelial cells secrete the chemokine interleukin-8 in response to bacterial entry. Infect. Immun. 61: 4569-4574.

Eglinton,B.A., Roberton,D.M., and Cummins,A.G. 1994. Phenotype of T cells, their soluble receptor levels, and cytokine profile of human breast milk. Immunol. Cell Biol. 72: 306-313.

Elango,R., Pencharz,P.B., and Ball,R.O. 2002. The branched-chain amino acid requirement of parenterally fed neonatal piglets is less than the enteral requirement. J. Nutr. 132: 3123-3129.

Fairbrother,J.M., Lariviere,S., and Lallier,R. 1986. New fimbrial antigen F165 from Escherichia coli serogroup O115 strains isolated from piglets with diarrhea. Infect. Immun. 51: 10-15.

Field,C.J., Johnson,I., and Pratt,V.C. 2000a. Glutamine and arginine: immunonutrients for improved health. Med. Sci. Sports Exerc. 32: S377-S388.

Field,C.J., Thomson,C.A., Van Aerde,J.E., Parrott,A., Euler,A., Lien,E., and Clandinin,M.T. 2000b. Lower proportion of CD45R0+ cells and deficient interleukin-10 production by formula-fed infants, compared with human-fed, is corrected with supplementation of long-chain polyunsaturated fatty acids. J. Pediatr. Gastroenterol. Nutr. 31: 291-299.

Field,C.J., Johnson,I.R., and Schley,P.D. 2002. Nutrients and their role in host resistance to infection. J. Leukoc. Biol. 71: 16-32.

Field,M. 1976. Regulation of active ion transport in the small intestine. Ciba Found. Symp. 109-127.

Fong,Y.M., Marano,M.A., Moldawer,L.L., Wei,H., Calvano,S.E., Kenney,J.S., Allison,A.C., Cerami,A., Shires,G.T., and Lowry,S.F. 1990. The acute splanchnic and peripheral tissue metabolic response to endotoxin in humans. J. Clin. Invest 85: 1896-1904.

Fortin,A., Robertson,W.M., Kibite,S., and Landry,S.J. 2003. Growth performance, carcass and pork quality of finisher pigs fed oat-based diets containing different levels of beta-glucans. J. Anim Sci. 81: 449-456.

Gaskins,R. 1998. Immunology and Neontal Mortality. In: The Neonatal Pig: Development and Survival. Varley,M.A. ed. Cab International. pp 39-55

Gebert,A., Rothkotter,H.J., and Pabst,R. 1996. M cells in Peyer's patches of the intestine. Int. Rev. Cytol. 167: 91-159.

Gianotti,L., Alexander,J.W., Gennari,R., Pyles,T., and Babcock,G.F. 1995. Oral glutamine decreases bacterial translocation and improves survival in experimental gut-origin sepsis. JPEN J. Parenter. Enteral Nutr. 19: 69-74.

Glass,R.I., Bresee,J., Jiang,B., Gentsch,J., Ando,T., Fankhauser,R., Noel,J., Parashar,U., Rosen,B., and Monroe,S.S. 2001. Gastroenteritis viruses: an overview. Novartis. Found. Symp. 238: 5-19.

Glezen,W.P. 2003. Effect of maternal antibodies on the infant immune response. Vaccine 21: 3389-3392.

Gonzalez-Amaro,R. and Sanchez-Madrid,F. 1999. Cell adhesion molecules: selectins and integrins. Crit Rev. Immunol. 19: 389-429.

Grange,P.A. and Mouricout,M.A. 1996. Transferrin associated with the porcine intestinal mucosa is a receptor specific for K88ab fimbriae of Escherichia coli. Infect. Immun. 64: 606-610.

Grange,P.A., Erickson,A.K., Anderson,T.J., and Francis,D.H. 1998. Characterization of the carbohydrate moiety of intestinal mucin-type sialoglycoprotein receptors for the K88ac fimbrial adhesin of Escherichia coli. Infect. Immun. 66: 1613-1621.

Grange,P.A., Erickson,A.K., Levery,S.B., and Francis,D.H. 1999. Identification of an intestinal neutral glycosphingolipid as a phenotype-specific receptor for the K88ad fimbrial adhesin of Escherichia coli. Infect. Immun. 67: 165-172.

Gyles,C.L. 1992. Escherichia coli cytotoxins and enterotoxins. Can. J. Microbiol. 38: 734-746.

Haks,M.C., Oosterwegel,M.A., Blom,B., Spits,H.M., and Kruisbeek,A.M. 1999. Cell-fate decisions in early T cell development: regulation by cytokine receptors and the pre-TCR. Semin. Immunol. 11: 23-37.

Haverson,K., Bailey,M., and Stokes,C.R. 1999. T-cell populations in the pig intestinal lamina propria: memory cells with unusual phenotypic characteristics. Immunology 96: 66-73.

Heyman,M. and Desjeux,J.F. 2000. Cytokine-induced alteration of the epithelial barrier to food antigens in disease. Ann. N. Y. Acad. Sci. 915: 304-311.

Hiss,S. and Sauerwein,H. 2003. Influence of dietary ss-glucan on growth performance, lymphocyte proliferation, specific immune response and haptoglobin plasma concentrations in pigs. J. Anim Physiol Anim Nutr. (Berl) 87: 2-11.

Hofman,P., Piche,M., Far,D.F., Le Negrate,G., Selva,E., Landraud,L., Alliana-Schmid,A., Boquet,P., and Rossi,B. 2000. Increased Escherichia coli phagocytosis in neutrophils that have transmigrated across a cultured intestinal epithelium. Infect. Immun. 68: 449-455.

Holmgren,J. 1981. Actions of cholera toxin and the prevention and treatment of cholera. Nature 292: 413-417.

Holtug,K., Hansen,M.B., and Skadhauge,E. 1996. Experimental studies of intestinal ion and water transport. Scand. J. Gastroenterol. Suppl 216: 95-110.

Holzmann,B. and Weissman,I.L. 1989. Integrin molecules involved in lymphocyte homing to Peyer's patches. Immunol. Rev. 108: 45-61.

Hornsby-Lewis,L., Shike,M., Brown,P., Klang,M., Pearlstone,D., and Brennan,M.F. 1994. L-glutamine supplementation in home total parenteral nutrition patients: stability, safety, and effects on intestinal absorption. JPEN J. Parenter. Enteral Nutr. 18: 268-273.

Hoskinson,C.D., Chew,B.P., and Wong,T.S. 1990. Age-related changes in mitogen-induced lymphocyte proliferation and polymorphonuclear neutrophil function in the piglet. J. Anim Sci. 68: 2471-2478.

House,J.D., Pencharz,P.B., and Ball,R.O. 1997. Phenylalanine requirements determined by using L-[1-14C]phenylalanine in neonatal piglets receiving total parenteral nutrition supplemented with tyrosine. Am. J. Clin. Nutr. 65: 984-993.

House,J.D., Pencharz,P.B., and Ball,R.O. 1998. Lysine requirement of neonatal piglets receiving total parenteral nutrition as determined by oxidation of the indicator amino acid L-[1-14C]phenylalanine. Am. J. Clin. Nutr. 67: 67-73.

Insoft,R.M., Sanderson,I.R., and Walker,W.A. 1996. Development of immune function in the intestine and its role in neonatal diseases. Pediatr. Clin. North Am. 43: 551-571.

Izydorczyk,M.S., Storsley,J., Labossiere,D., MacGregor,A.W., and Rossnagel,B.G. 2000. Variation in total and soluble beta-glucan content in hulless barley: effects of thermal, physical, and enzymic treatments. J. Agric. Food Chem. 48: 982-989.

Jaye,D.L. and Parkos,C.A. 2000. Neutrophil migration across intestinal epithelium. Ann. N. Y. Acad. Sci. 915: 151-161.

Jensen,G.L., Miller,R.H., Talabiska,D.G., Fish,J., and Gianferante,L. 1996. A double-blind, prospective, randomized study of glutamine-enriched compared with standard peptide-based feeding in critically ill patients. American Journal of Clinical Nutrition 64: 615-621.

Jepson,M.A. and Clark,M.A. 1998. Studying M cells and their role in infection. Trends Microbiol. 6: 359-365.

Jiang,G. and Vasanthan,T. 2000. MALDI-MS and HPLC quantification of oligosaccharides of lichenase-hydrolyzed water-soluble beta-glucan from ten barley varieties. J. Agric. Food Chem. 48: 3305-3310.

Jin,L.Z. and Zhao,X. 2000. Intestinal receptors for adhesive fimbriae of enterotoxigenic Escherichia coli (ETEC) K88 in swine--a review. Appl. Microbiol. Biotechnol. 54: 311-318.

Johnson,A.T., Kaufmann,Y.C., Luo,S., Todorova,V., and Klimberg,V.S. 2003. Effect of glutamine on glutathione, IGF-I, and TGF-beta 1. J. Surg. Res. 111: 222-228.

Jonjic,N., Jonjic,S., Saalmuller,A., Rukavina,D., and Koszinowski,U.H. 1987. Distribution of T-lymphocyte subsets in porcine lymphoid tissues. Immunology 60: 395-401.

Kantele,J.M., Arvilommi,H., Kontiainen,S., Salmi,M., Jalkanen,S., Savilahti,E., Westerholm,M., and Kantele,A. 1996. Mucosally activated circulating human B cells in diarrhea express homing receptors directing them back to the gut. Gastroenterology 110: 1061-1067.

Keely, S.J., and Barrett,K.E. 2000. Regulation of chloride secretion: novel pathways and messengers. Ann. N. Y. Acad. Sci. 915: 67-76.

Keren,D.F. 1992. Antigen processing in the mucosal immune system. Semin. Immunol. 4: 217-226.

Khan,A.S., Johnston,N.C., Goldfine,H., and Schifferli,D.M. 1996. Porcine 987P glycolipid receptors on intestinal brush borders and their cognate bacterial ligands. Infect. Immun. 64: 3688-3693.

Klimberg,V.S., Salloum,R.M., Kasper,M., Plumley,D.A., Dolson,D.J., Hautamaki,R.D., Mendenhall,W.R., Bova,F.C., Bland,K.I., Copeland,E.M., III, and Souba,W.W. 1990. Oral Glutamine Accelerates Healing of the Small Intestine and Improves Outcome After Whole Abdominal Radiation. Archives of Surgery 125: 1040-1045.

Klobasa,F., Werhahn,E., and Butler,J.E. 1981. Regulation of humoral immunity in the piglet by immunoglobulins of maternal origin. Res. Vet. Sci. 31: 195-206.

Klobasa,F., Butler,J.E., Werhahn,E., and Habe,F. 1986. Maternal-neonatal immunoregulation in swine. II. Influence of multiparity on de novo immunoglobulin synthesis by piglets. Vet. Immunol. Immunopathol. 11: 149-159.

Koefoed-Johnsen,V. and Ussing,H.H. 1958. The nature of the frog skin potential. Acta Physiol Scand. 42: 298-308.

Kovarik,J. and Siegrist,C.A. 1998. Immunity in early life. Immunol. Today 19: 150-152.

Krebs,H.A., Baverel,G., and Lund,P. 1980. Effect of bicarbonate on glutamine metabolism. Int. J. Biochem. 12: 69-73.

Laissue,J.A., Chappuis,B.B., Muller,C., Reubi,J.C., and Gebbers,J.O. 1993. The intestinal immune system and its relation to disease. Dig. Dis. 11: 298-312.

Lang,C.H., Obih,J.A., Bagby,G.J., Bagwell,J.N., and Spitzer,J.J. 1991. Endotoxin-induced increases in regional glucose utilization by small intestine: a TNF-independent effect. Am. J. Physiol 260: G548-G555.

Langkamp-Henken,B., Glezer,J.A., and Kudsk,K.A. 1992. Immunologic structure and function of the gastrointestinal tract. Nutr. Clin. Pract. 7: 100-108.

Laux,D.C., McSweegan,E.F., Williams,T.J., Wadolkowski,E.A., and Cohen,P.S. 1986. Identification and characterization of mouse small intestine mucosal receptors for Escherichia coli K-12(K88ab). Infect. Immun. 52: 18-25.

Lebron,F., Vassallo,R., Puri,V., and Limper,A.H. 2003. Pneumocystis carinii cell wall beta-glucans initiate macrophage inflammatory responses through NF-kappaB activation. J. Biol. Chem. 278: 25001-25008.

Lecce,J.G. 1975. Rearing piglets artificially in a farm environment: a promise unfulfilled. J. Anim Sci. 41: 659-666.

Levine,M.M. and Edelman,R. 1984. Enteropathogenic Escherichia coli of classic serotypes associated with infant diarrhea: epidemiology and pathogenesis. Epidemiol. Rev. 6: 31-51.

Levine,M.M. and Noriega,F. 1993. Vaccines to prevent enteric infections. Baillieres Clin. Gastroenterol. 7: 501-517.

Lunney,J.K. and Pescovitz,M.D. 1987. Phenotypic and functional characterization of pig lymphocyte populations. Vet. Immunol. Immunopathol. 17: 135-144.

MacDonald,M.L., Rogers,Q.R., and Morris,J.G. 1984. Nutrition of the domestic cat, a mammalian carnivore. Annu. Rev. Nutr. 4: 521-562.

Madara,J.L. and Stafford,J. 1989. Interferon-gamma directly affects barrier function of cultured intestinal epithelial monolayers. J. Clin. Invest 83: 724-727.

Malek,T.R. 2003. The main function of IL-2 is to promote the development of T regulatory cells. J. Leukoc. Biol. 74: 961-965.

Mannick,E. and Udall,J.N., Jr. 1996. Neonatal gastrointestinal mucosal immunity. Clin. Perinatol. 23: 287-304.

Mattioli,C.A. and Tomasi,T.B., Jr. 1973. The life span of IgA plasma cells from the mouse intestine. J. Exp. Med. 138: 452-460.

McCauley,I. and Hartmann,P.E. 1984. Changes in piglet leucocytes, B lymphocytes and plasma cortisol from birth to three weeks after weaning. Res. Vet. Sci. 37: 234-241.

McCormick,B.A., Colgan,S.P., Delp-Archer,C., Miller,S.I., and Madara,J.L. 1993. Salmonella typhimurium attachment to human intestinal epithelial monolayers: transcellular signalling to subepithelial neutrophils. J. Cell Biol. 123: 895-907.

McCormick,B.A., Parkos,C.A., Colgan,S.P., Carnes,D.K., and Madara,J.L. 1998. Apical secretion of a pathogen-elicited epithelial chemoattractant activity in response to surface colonization of intestinal epithelia by Salmonella typhimurium. J. Immunol. 160: 455-466.

McIntosh,G.H., Whyte,J., McArthur,R., and Nestel,P.J. 1991. Barley and wheat foods: influence on plasma cholesterol concentrations in hypercholesterolemic men. Am. J. Clin. Nutr. 53: 1205-1209.

McKay,D.M. and Perdue,M.H. 1993. Intestinal epithelial function: the case for immunophysiological regulation. Cells and mediators (1). Dig. Dis. Sci. 38: 1377-1387.

McWilliams,M., Phillips-Quagliata,J.M., and Lamm,M.E. 1977. Mesenteric lymph node B lymphoblasts which home to the small intestine are precommitted to IgA synthesis. J. Exp. Med. 145: 866-875.

Miller,B.G., Newby,T.J., Stokes,C.R., and Bourne,F.J. 1984. Influence of diet on postweaning malabsorption and diarrhoea in the pig. Res. Vet. Sci. 36: 187-193.

Miyake,K. 2003. Innate recognition of lipopolysaccharide by CD14 and toll-like receptor 4-MD-2: unique roles for MD-2. Int. Immunopharmacol. 3: 119-128.

Mobert,J., Zahler,S., Becker,B.F., and Conzen,P.F. 1999. Inhibition of neutrophil activation by volatile anesthetics decreases adhesion to cultured human endothelial cells. Anesthesiology 90: 1372-1381.

Moon,H.W. 1978. Mechanisms in the pathogenesis of diarrhea: a review. J. Am. Vet. Med. Assoc. 172: 443-448.

Moughan,P.J., Birtles,M.J., Cranwell,P.D., Smith,W.C., and Pedraza,M. 1992. The piglet as a model animal for studying aspects of digestion and absorption in milk-fed human infants. World Rev. Nutr. Diet. 67: 40-113.

Murphy,C. and Newsholme,P. 1998. Importance of glutamine metabolism in murine macrophages and human monocytes to L-arginine biosynthesis and rates of nitrite or urea production. Clin. Sci. 95: 397-407.

Mrsny,R.J., Gewirtz,A.T., Siccardi,D., Savidge,T., Hurley,B.P., Madara,J.L., and McCormick,B.A. 2004. Identification of hepoxilin A_3 in inflammatory events: A required role in neutrophil migration across intestinal epithelia. Proc. Natl. Acad. Sci. U.S.A. 101: 7421-7426.

Nabuurs,M.J. 1998. Weaning piglets as a model for studying pathophysiology of diarrhea. Vet. Q. 20 Suppl 3: S42-S45.

Nagy,B., Casey,T.A., Whipp,S.C., and Moon,H.W. 1992. Susceptibility of porcine intestine to pilus-mediated adhesion by some isolates of piliated enterotoxigenic Escherichia coli increases with age. Infect. Immun. 60: 1285-1294.

Newsholme,E.A., Crabtree,B., and Ardawi,M.S. 1985. The role of high rates of glycolysis and glutamine utilization in rapidly dividing cells. Biosci. Rep. 5: 393-400.

Newsholme,E.A. and Parry-Billings,M. 1990. Properties of Glutamine Release From Muscle and Its Importance for the Immune System. Journal of Parenteral and Enteral Nutrition 14: 63S-67S.

Newsholme,E.A. and Calder,P.C. 1997. The proposed role of glutamine in some cells of the immune system and speculative consequences for the whole animal. Nutrition 13: 728-730.

Ngo,A., Coon,C.N., and Beecher,G.R. 1977. Dietary glycine requirements for growth and cellular development in chicks. J. Nutr. 107: 1800-1808.

Novak,D.A. and Beveridge,M.J. 1997. Glutamine transport in human and rat placenta. Placenta 18: 379-386.

NRC 1998. Nutrient Requirements of swine (10th Ed.). Subcom. Swine Nutr., Comm Anim. Nutr., Board Agric., NRC. Nat. Acad. Press, Wash. pp 192.

Nusrat,A., Turner,J.R., and Madara,J.L. 2000. Molecular physiology and pathophysiology of tight junctions. IV. Regulation of tight junctions by extracellular stimuli: nutrients, cytokines, and immune cells. Am. J. Physiol Gastrointest. Liver Physiol 279: G851-G857.

O'Dowd,Y. and Newsholme,P. 1997. Evidence for the involvement of glutamine in nitric oxide (NO) production by immunostimulated neutrophils. Biochem. Soc. Trans. 25: 403S.

Ogle,C.K., Ogle,J.D., Mao,J.-X., Simon,J., Noel,J.G., Li,B.-G., and Alexander,J.W. 1994. Effect of Glutamine on Phagocytosis and Bacterial Killing by Normal and Pediatric Burn Patient Neutrophils. Journal of Parenteral and Enteral Nutrition 18: 128-133.

Owen,R.L. and Jones,A.L. 1974. Epithelial cell specialization within human Peyer's patches: an ultrastructural study of intestinal lymphoid follicles. Gastroenterology 66: 189-203.

Owen,R.L. 1977. Sequential uptake of horseradish peroxidase by lymphoid follicle epithelium of Peyer's patches in the normal unobstructed mouse intestine: an ultrastructural study. Gastroenterology 72: 440-451.

Pabst,R., Geist,M., Rothkotter,H.J., and Fritz,F.J. 1988. Postnatal development and lymphocyte production of jejunal and ileal Peyer's patches in normal and gnotobiotic pigs. Immunology 64: 539-544.

Pabst,R. and Rothkotter,H.J. 1999. Postnatal development of lymphocyte subsets in different compartments of the small intestine of piglets. Vet. Immunol. Immunopathol. 72: 167-173.

Paniagua,M., Espinoza,F., Ringman,M., Reizenstein,E., Svennerholm,A.M., and Hallander,H. 1997. Analysis of incidence of infection with enterotoxigenic Escherichia coli in a prospective cohort study of infant diarrhea in Nicaragua. J. Clin. Microbiol. 35: 1404-1410.

Parry-Billings,M., Evans,J., Calder,P.C., and Newsholme,E.A. 1990. Does glutamine contribute to immunosuppression after major burns? Lancet 336: 523-525.

Patel,S.S., Wacholtz,M.C., Duby,A.D., Thiele,D.L., and Lipsky,P.E. 1989. Analysis of the functional capabilities of CD3+CD4-CD8- and CD3+CD4+CD8+ human T cell clones. J. Immunol. 143: 1108-1117.

Patience,J.F. and Thacker,P.A. 1989. Swine Nutrition Guide. Univ. Saskat. and Prairie Swine Ctr, Saskatoon, Sask. pp 260.

Patra,F.C., Sack,D.A., Islam,A., Alam,A.N., and Mazumder,R.N. 1989. Oral rehydration formula containing alanine and glucose for treatment of diarrhoea: a controlled trial. BMJ 298: 1353-1356.

Pencharz,P.B. and Ball,R.O. 2003. Different approaches to define individual amino acid requirements. Annu. Rev. Nutr. 23: 101-116.

Pescovitz,M.D., Lunney,J.K., and Sachs,D.H. 1985. Murine anti-swine T4 and T8 monoclonal antibodies: distribution and effects on proliferative and cytotoxic T cells. J. Immunol. 134: 37-44.

Pescovitz,M.D., Hsu,S.M., Katz,S.I., Lunney,J.K., Shimada,S., and Sachs,D.H. 1990. Characterization of a porcine CD1-specific mAb that distinguishes CD4/CD8 double-positive thymic from peripheral T lymphocytes. Tissue Antigens 35: 151-156.

Pescovitz,M.D., Sakopoulos,A.G., Gaddy,J.A., Husmann,R.J., and Zuckermann,F.A. 1994. Porcine peripheral blood CD4+/CD8+ dual expressing T-cells. Vet. Immunol. Immunopathol. 43: 53-62.

Pescovitz,M.D., Book,B.K., Aasted,B., Dominguez,J., Ezquerra,A., Trebichavsky,I., Novikov,B., Valpotic,I., Nielsen,J., Arn,S., Sachs,D.H., Lunney,J.K., Boyd,P.C., Walker,J., Lee,R., Lackovic,G., Kirkham,P., Parkhouse,R.M., and Saalmuller,A. 1998. Analyses of monoclonal antibodies reacting with porcine CD3: results from the Second International Swine CD Workshop. Vet. Immunol. Immunopathol. 60: 261-268.

Peskar,B.M., Weiler,H., Kroner,E.E., and Peskar,B.A. 1981. Release of prostaglandins by small intestinal tissue of man and rat in vitro and the effect of endotoxin in the rat in vivo. Prostaglandins 21 Suppl: 9-14.

Quiding-Jarbrink,M., Lakew,M., Nordstrom,I., Banchereau,J., Butcher,E., Holmgren,J., and Czerkinsky,C. 1995. Human circulating specific antibody-forming cells after systemic and mucosal immunizations: differential homing commitments and cell surface differentiation markers. Eur. J. Immunol. 25: 322-327.

Rerat,A. 1978. Digestion and absorption of carbohydrates and nitrogenous matters in the hindgut of the omnivorous nonruminant animal. J. Anim Sci. 46: 1808-1837.

Riggi,S.J. and Di Luzio,N.R. 1961. Identification of a reticuloendothelial stimulating agent in zymosan. Am. J. Physiol 200: 297-300.

Robinson,L.E., Bussiere,F.I., Le Boucher,J., Farges,M.C., Cynober,L.A., Field,C.J., and Baràcos,V.E. 1999. Amino acid nutrition and immune function in tumour-bearing rats: a comparison of glutamine-, arginine- and ornithine 2-oxoglutarate-supplemented diets. Clin. Sci. (Lond) 97: 657-669.

Rombeau,J.L. 1990. A review of the effects of glutamine-enriched diets on experimentally induced enterocolitis. JPEN J. Parenter. Enteral Nutr. 14: 100S-105S.

Rosenberg,Y.J., Anderson,A.O., and Pabst,R. 1998. HIV-induced decline in blood CD4/CD8 ratios: viral killing or altered lymphocyte trafficking? Immunol. Today 19: 10-17.

Rothkotter,H.J. and Pabst,R. 1989. Lymphocyte subsets in jejunal and ileal Peyer's patches of normal and gnotobiotic minipigs. Immunology 67: 103-108.

Rothkotter,H.J., Ulbrich,H., and Pabst,R. 1991. The postnatal development of gut lamina propria lymphocytes: number, proliferation, and T and B cell subsets in conventional and germ-free pigs. Pediatr. Res. 29: 237-242.

Rothkotter,H.J., Huber,T., Barman,N.N., and Pabst,R. 1993. Lymphoid cells in afferent and efferent intestinal lymph: lymphocyte subpopulations and cell migration. Clin. Exp. Immunol. 92: 317-322.

Rothkotter,H.J., Pabst,R., and Bailey,M. 1999. Lymphocyte migration in the intestinal mucosa: entry, transit and emigration of lymphoid cells and the influence of antigen. Vet. Immunol. Immunopathol. 72: 157-165.

Ruggeberg,J., Stalmach,E., Zubrod-Eichert,C., Wahn,V., and Schroten,H. 1997. Antimicrobial Functions of Human Monocytes Depend on Concentration of glutamine in vitro. Annals of Nutrition Metabolism 41: 371-375.

Saalmuller,A., Jonjic,S., Buhring,H.J., Reddehase,M.J., and Koszinowski,U.H. 1987a. Monoclonal antibodies reactive with swine lymphocytes. II. Detection of an antigen on resting T cells down-regulated after activation. J. Immunol. 138: 1852-1857.

Saalmuller,A., Reddehase,M.J., Buhring,H.J., Jonjic,S., and Koszinowski,U.H. 1987b. Simultaneous expression of CD4 and CD8 antigens by a substantial proportion of resting porcine T lymphocytes. Eur. J. Immunol. 17: 1297-1301.

Saalmuller,A., Hirt,W., and Reddehase,M.J. 1989. Phenotypic discrimination between thymic and extrathymic CD4-CD8- and CD4+CD8+ porcine T lymphocytes. Eur. J. Immunol. 19: 2011-2016.

Saalmuller,A. and Bryant,J. 1994. Characteristics of porcine T lymphocytes and T-cell lines. Vet. Immunol. Immunopathol. 43: 45-52.

Salmon,H. 1987. The intestinal and mammary immune system in pigs. Vet. Immunol. Immunopathol. 17: 367-388.

Sandborg,R.R. and Smolen,J.E. 1988. Early biochemical events in leukocyte activation. Lab Invest 59: 300-320.

Sarmiento,J.I., Casey,T.A., and Moon,H.W. 1988. Postweaning diarrhea in swine: experimental model of enterotoxigenic Escherichia coli infection. Am. J. Vet. Res. 49: 1154-1159.

Savidge,T.C. 1996. The life and times of an intestinal M cell. Trends Microbiol. 4: 301-306.

Schley,P.D. and Field,C.J. 2002. The immune-enhancing effects of dietary fibres and prebiotics. Br. J. Nutr. 87 Suppl 2: S221-S230.

Schneeman,B.O. 1999. Fiber, inulin and oligofructose: similarities and differences. J. Nutr. 129: 1424S-1427S.

Schoenherr,W.D., Pollmann,D.S., and Coalson,J.A. Titration of MacroGard-S on growth performance of nursery pigs. J.Anim Sci. 72 (suppl. 2), 57. 1994.

Sellwood,R. 1979. Escherichia coli diarrhoea in pigs with or without the K88 receptor. Vet. Rec. 105: 228-230.

Shipley,S.G. 1996. Glutamine in Total Parenteral Nutrition. Nutrition Today 31: 74-77.

Shoveller,A.K., Brunton,J.A., Pencharz,P.B., and Ball,R.O. 2003. The methionine requirement is lower in neonatal piglets fed parenterally than in those fed enterally. J. Nutr. 133: 1390-1397.

Siebers,A. and Finlay,B.B. 1996. M cells and the pathogenesis of mucosal and systemic infections. Trends Microbiol. 4: 22-29.

Sinkora,J., Rehakova,Z., Sinkora,M., Cukrowska,B., and Tlaskalova-Hogenova,H. 2002. Early development of immune system in pigs. Vet. Immunol. Immunopathol. 87: 301-306.

Skansen-Saphir,U., Lindfors,A., and Andersson,U. 1993. Cytokine production in mononuclear cells of human milk studied at the single-cell level. Pediatr. Res. 34: 213-216.

Slifka,M.K., Antia,R., Whitmire,J.K., and Ahmed,R. 1998. Humoral immunity due to long-lived plasma cells. Immunity. 8: 363-372.

Smith,R.J. 1990. Glutamine Metabolism and Its Physiological Importance. Journal of Parenteral and Enteral Nutrition 14: 40S-44S.

Souba,W.W. 1993. Intestinal glutamine metabolism and nutrition. Journal of Nutritional Biochemistry 4: 2-9.

Souba,W.W. and Austgen,T.R. 1990. Interorgan Glutamine Flow Following Surgery and Infection. Journal of Parenteral and Enteral Nutrition 14: 90S-93S.

Spreeuwenberg,M.A., Verdonk,J.M., Gaskins,H.R., and Verstegen,M.W. 2001. Small intestine epithelial barrier function is compromised in pigs with low feed intake at weaning. J. Nutr. 131: 1520-1527.

Springer,T.A. 1994. Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. Cell 76: 301-314.

Springer,T.A. 1995. Traffic signals on endothelium for lymphocyte recirculation and leukocyte emigration. Annu. Rev. Physiol 57: 827-872.

Stokes,C.R., Bailey,M., and Wilson,A.D. 1994. Immunology of the porcine gastrointestinal tract. Vet. Immunol. Immunopathol. 43: 143-150.

Stokes,C.R., Haverson,K., and Bailey,M. 1996. Antigen presenting cells in the porcine gut. Vet. Immunol. Immunopathol. 54: 171-177.

Su,T.Z., Campbell,G.W., and Oxender,D.L. 1997. Glutamine transport in cerebellar granule cells in culture. Brain Res. 757: 69-78.

Summerfield,A., Rziha,H.J., and Saalmuller,A. 1996. Functional characterization of porcine CD4+CD8+ extrathymic T lymphocytes. Cell Immunol. 168: 291-296.

Sun,X.M. and Hsueh,W. 1988. Bowel necrosis induced by tumor necrosis factor in rats is mediated by platelet-activating factor. J. Clin. Invest 81: 1328-1331.

Sundberg,B., Wood,P., Lia,A., Andersson,H., Sandberg,A.S., Hallmans,G., and Aman,P. 1996. Mixed-linked beta-glucan from breads of different cereals is partly degraded in the human ileostomy model. Am. J. Clin. Nutr. 64: 878-885.

Szabo,S.J., Sullivan,B.M., Peng,S.L., and Glimcher,L.H. 2003. Molecular mechanisms regulating Th1 immune responses. Annu. Rev. Immunol. 21: 713-758.

Szondy,Z. and Newsholme,E.A. 1989. The effect of glutamine concentration on the activity of carbamoyl-phosphate synthase II and on the incorporation of [3H]thymidine into DNA in rat mesenteric lymphocytes stimulated by phytohaemagglutinin. Biochem. J. 261: 979-983.

Tanimoto,T. and Ohtsuki,Y. 1996. Evaluation of antibodies reactive with porcine lymphocytes and lymphoma cells in formalin-fixed, paraffin-embedded, antigen-retrieved tissue sections. Am. J. Vet. Res. 57: 853-859.

Tayeh,M.A. and Marletta,M.A. 1989. Macrophage oxidation of L-arginine to nitric oxide, nitrite, and nitrate. Tetrahydrobiopterin is required as a cofactor. J. Biol. Chem. 264: 19654-19658.

Thorns,C.J., Sojka,M.G., and Roeder,P.L. 1989. Detection of fimbrial adhesins of ETEC using monoclonal antibody-based latex reagents. Vet. Rec. 125: 91-92.

Tlaskalova-Hogenova,H., Mandel,L., Trebichavsky,I., Kovaru,F., Barot,R., and Sterzl,J. 1994. Development of immune responses in early pig ontogeny. Vet. Immunol. Immunopathol. 43: 135-142.

Touchette,K.J., Carroll,J.A., Allee,G.L., Matteri,R.L., Dyer,C.J., Beausang,L.A., and Zannelli,M.E. 2002. Effect of spray-dried plasma and lipopolysaccharide exposure on weaned pigs: I. Effects on the immune axis of weaned pigs. J. Anim Sci. 80: 494-501.

Travnicek,J., Mandel,L., Trebichavsky,I., and Talafantova,M. 1989. Immunological state of adult germfree miniature Minnesota pigs. Folia Microbiol. (Praha) 34: 157-164.

Ulevitch,R.J. and Tobias,P.S. 1995. Receptor-dependent mechanisms of cell stimulation by bacterial endotoxin. Annu. Rev. Immunol. 13: 437-457.

Ussing,H.H. and Zerahn,K. 1951. Active transport of sodium as the source of electric current in the short-circuited isolated frog skin. Acta Physiol Scand. 23: 110-127.

Ussing,H.H. 1994. Does active transport exist? J. Membr. Biol. 137: 91-98.

Uzzau,S. and Fasano,A. 2000. Cross-talk between enteric pathogens and the intestine. Cell Microbiol. 2: 83-89.

Valpotic,I., Gerencer,M., and Basic,I. 1989. In vitro modulating effects of porcine immunoglobulin G on mitogens-induced lymphocyte response in precolostral, suckling and weaned piglets. Vet. Immunol. Immunopathol. 22: 113-122.

van Acker,B.A., von Meyenfeldt,M.F., van der Hulst,R.R., Hulsewe,K.W., Wagenmakers,A.J., Deutz,N.E., de,B., I, Dejong,C.H., van Kreel,B.K., and Soeters,P.B. 1999. Glutamine: the pivot of our nitrogen economy? JPEN J Parenter. Enteral Nutr 23: S45-S48.

van der Hulst,R.R., von Meyenfeldt,M.F., Tiebosch,A., Buurman,W.A., and Soeters,P.B. 1997. Glutamine and intestinal immune cells in humans. JPEN J Parenter. Enteral Nutr 21: 310-315.

van der Merwe,P.A. and Davis,S.J. 2003. Molecular interactions mediating T cell antigen recognition. Annu. Rev. Immunol. 21: 659-684.

Velazquez,O.C., Lederer,H.M., and Rombeau,J.L. 1997. Butyrate and the colonocyte: production, absorption, and treatment of hyperlipidaemias. In: Dietary Fibre - A Component of Food. Schweizer,T.F. and Edwards,C.A. eds. Springer-Verlag, London, UK. pp 295-332.

Vega-Lopez,M.A., Bailey,M., Telemo,E., and Stokes,C.R. 1995. Effect of early weaning on the development of immune cells in the pig small intestine. Vet. Immunol. Immunopathol. 44: 319-327.

Vina,J., Vento,M., Garcia-Sala,F., Puertes,I.R., Gasco,E., Sastre,J., Asensi,M., and Pallardo,F.V. 1995. L-cysteine and glutathione metabolism are impaired in premature infants due to cystathionase deficiency. Am. J. Clin. Nutr. 61: 1067-1069.

Webel,D.M., Finck,B.N., Baker,D.H., and Johnson,R.W. 1997. Time course of increased plasma cytokines, cortisol, and urea nitrogen in pigs following intraperitoneal injection of lipopolysaccharide. J. Anim Sci. 75: 1514-1520.

Weiss,A. 1993. T cell antigen receptor signal transduction: a tale of tails and cytoplasmic protein-tyrosine kinases. Cell 73: 209-212.

Whary,M.T., Zarkower,A., Confer,F.L., and Ferguson,F.G. 1995. Age-related differences in subset composition and activation responses of intestinal intraepithelial and mesenteric lymph node lymphocytes from neonatal swine. Cell Immunol. 163: 215-221.

Willment,J.A., Gordon,S., and Brown,G.D. 2001. Characterization of the human β-blucan receptor and its alternatively spliced isoforms. J Biol Chem 276: 43818-43823.

Windmueller,H.G. and Spaeth,A.E. 1974. Uptake and metabolism of plasma glutamine by the small intestine. J Biol. Chem. 249: 5070-5079.

Woodward,B.D., Bezanson,K.D., Hillyer,L.M., and Lee,W.H. 1995. The CD45RA+ (quiescent) cellular phenotype is overabundant relative to the CD. J. Nutr. 125: 2471-2482.

Wu,G.Y., Field,C.J., and Marliss,E.B. 1991a. Glucose and glutamine metabolism in rat macrophages: enhanced glycolysis and unaltered glutaminolysis in spontaneously diabetic BB rats. Biochim. Biophys. Acta 1115: 166-173.

Wu,G.Y., Field,C.J., and Marliss,E.B. 1991b. Glutamine and glucose metabolism in thymocytes from normal and spontaneously diabetic BB rats. Biochem. Cell Biol. 69: 801-808.

Wu,G., Field,C.J., and Marliss,E.B. 1992. Enhanced glutamine and glucose metabolism in cultured rat splenocytes stimulated by phorbol myristate acetate plus ionomycin. Metabolism 41: 982-988.

Wu,G. and Meininger,C.J. 1993. Regulation of L-arginine synthesis from L-citrulline by L-glutamine in endothelial cells. Am. J. Physiol 265: H1965-H1971.

Wu,G. and Knabe,D.A. 1994. Free and protein-bound amino acids in sow's colostrum and milk. J Nutr 124: 415-424.

Wu,G., Knabe,D.A., and Flynn,N.E. 1994. Synthesis of citrulline from glutamine in pig enterocytes. Biochem. J 299 (Pt 1): 115-121.

Wu,G., Meier,S.A., and Knabe,D.A. 1996. Dietary glutamine supplementation prevents jejunal atrophy in weaned pigs. J. Nutr. 126: 2578-2584.

Wu,G. 1998. Intestinal mucosal amino acid catabolism. J Nutr 128: 1249-1252.

Wykes,L.J., Ball,R.O., and Pencharz,P.B. 1993. Development and validation of a total parenteral nutrition model in the neonatal piglet. J. Nutr. 123: 1248-1259.

Yang,H. and Binns,R.M. 1993a. CD44 is not directly involved in the binding of lymphocytes to cultured high endothelial cells from peripheral lymph nodes. Immunology 79: 418-424.

Yang,H. and Binns,R.M. 1993b. Expression and regulation of the porcine CD44 molecule. Cell Immunol. 149: 117-129.

Yoo,S.S., Field,C.J., and McBurney,M.I. 1997. Glutamine supplementation maintains intramuscular glutamine concentrations and normalizes lymphocyte function in infected early weaned pigs. J Nutr 127: 2253-2259.

Zapata-Sirvent,R.L., Hansbrough,J.F., Ohara,M.M., Rice-Asaro,M., and Nyhan,W.L. 1994. Bacterial translocation in burned mice after administration of various diets including fiber- and glutamine-enriched enteral formulas. Crit Care Med. 22: 690-696.

Zhang,J.H., Li,D.F., Gong,L.M., Zhang,Y.C., and Zhang,D.F. 2002. Optimum ratio of histidine in the piglet ideal protein model and its effects on body metabolism. I. Basal diet formulation based on digestible amino acids according to the ideal protein model for 10 to 20 kg piglets. Arch. Tierernahr. 56: 109-116.

Zimmerman,D.R. 1975. Tryptophan requirements of 5- to 15-kg pigs with semipractical pig starters. J. Anim Sci. 40: 875-879.

Zuckermann,F.A. and Gaskins,H.R. 1996. Distribution of porcine CD4/CD8 double-positive T lymphocytes in mucosa-associated lymphoid tissues. Immunology 87: 493-499.

Zuckermann,F.A. and Husmann,R.J. 1996. Functional and phenotypic analysis of porcine peripheral blood CD4/CD8 double-positive T cells. Immunology 87: 500-512.

CHAPTER 2 - RESEARCH PLAN

2.1 RATIONALE

Enterotoxigenic *Escherichia coli* (ETEC) is a causative agent in approximately 800,000 cases of diarrhea in children under 5 years of age annually (Paniagua et al. 1997) and in piglets at weaning (Nabuurs 1998). As piglets are susceptible to ETEC infections and the process by which the organism infects them, they offer a logical model to determine the effect of diet on infant ETEC. Swine also grow and metabolize nutrients more similar to humans (Saalmuller et al. 1989) compared to mice, the most frequently used experimental model, which have a very short gestation and distance in phylogeny (Sinkora et al. 2002). Although there has been considerable research on the immune system in pigs, less is known about immune function during the weaning period.

The amino acid gln is a conditionally essential nutrient that becomes conditionally essential in many pathologic states (Smith 1990). The benefits of gln have been shown at several immune sites, including maintenance of the gut mucosa (Alverdy 1990; Wu et al. 1996), enhancing the function of several immune cell populations (Shipley 1996) and increasing the synthesis of growth factors and cytokines (Tayeh & Marletta 1989; Newsholme & Calder 1997; Ruggeberg et al. 1997). B-glc from various plants, bacteria, and fungi have been shown to bind to receptors on macrophages and other white blood cells, activating them (Brown & Gordon 2001), and promoting resistance to infections (Williams & Di Luzio 1979) and tumors *in vivo* (Chen & Hasumi 1993). *In vitro*, B-glc have been shown to influence adaptive immune function by increasing lymphocyte proliferation and cytotoxicity (Kim et al. 1996) and innate immune function components such as complement (Vetvicka et al. 1996) and neutrophil function (Zhang & Petty 1994; Willment et al. 2001). In conclusion, feeding these dietary nutrients has the potential to influence immune and gut development and support the immune system during ETEC infection.

2.2 OBJECTIVES AND HYPOTHESES

The overall aim of this research is to determine the efficacy of two dietary constituents (i.e. gln and B-glc) on immune and gut development in weaned piglets.

The objectives and hypotheses of this research are:

A. To develop an experimental model of ETEC infection in the piglet

It is hypothesized that:

1. Oral inoculation with live ETEC to weaned piglets will result in reproducible clinical symptoms of mild infection.

2. An *in vivo* loop model would demonstrate the early intestinal sings of ETEC infection. The early signs of infection are defined by a) intestinal response b) response of gut-associated lymphoid tissue (GALT).

B. To characterize changes during the 2 week post-weaning period on immune defense

It is hypothesized that:

3. Piglet innate immune defense at 35 days will be improved compared to that at 21 days. An improvement in innate immune defense was defined by increased neutrophil function.

4. Piglet adaptive immune defense at 35 days is more developed than at 21 days. Development of adaptive immune defense was defined as a) an increased response to mitogens in lymphocytes isolated from blood, mesenteric lymph nodes (MLN) and Peyer's patches (PP), b) an increased production of specific cytokines from these mitogen-stimulated lymphocytes, and c) changes in immune cell populations that reflect increased maturation.

C. To determine the ability of supplemental dietary gln (4.4 % w/w) to: a) promote immune function and defense against ETEC challenge and b) improve gastrointestinal morphology and function against an ETEC challenge in the early post-weaning period in piglets.

It is hypothesized that:

5. Supplementation of dietary gln to newly weaned piglets will improve innate immune defense. An improvement in innate immune defense was defined by increased neutrophil function.

6. Supplementation of dietary gln to newly weaned piglets will improve adaptive immune defense. Improved defense was defined as an increase in proliferative response (^{3}H-thymidine uptake and cytokine production) to mitogens by lymphocytes isolated from blood, MLN and PP.

7. Supplementation of gln to newly weaned piglets will benefit gut development in the newly weaned piglet. An improvement in gut development was defined as changes in jejunal permeability and electrophysiology measures.

8. Intestinal loops challenged with ETEC from gln-supplemented piglets will maintain a) intestinal barrier function and b) functional integrity. Intestinal functional integrity was assessed by measuring potential difference (PD), short-circuit current (Isc) and mannitol flux (ManP).

D. To determine the effect of feeding barley derived b-glc on a) immune function b) gastrointestinal function

It is hypothesized that:

9. Supplementation of dietary Barley-derived B-glc will improve innate immune defense. An improvement in innate immune defense was defined by increased neutrophil function and increased concentration of blood phagocytic cell numbers.

10. Supplementation of Barley-derived B-glc can improve adaptive immune defense. Improved immune defense was defined as an increase in proliferation in response to mitogens (^{3}H-thymidine uptake and cytokine production) by lymphocytes isolated from blood, MLN and PP.

11. Supplementation of Barley-derived B-glc will benefit gut development in newly weaned piglets, defined by gut permeability, absorption and secretion (Ussing chambers).

12. Supplementation of diets varying in Fiber and B-glc content will decrease the ability of isolated enterocytes to bind Fluorescein isothiocyanate- (FITC) labelled *Escherichia coli.*

2.3 CHAPTER FORMAT

The hypotheses posed are tested in a sequence of experiments. These experiments are organized in chapters as follows:

A. Chapter III will test Hypothesis 1 and 2

B. Chapter IV will test hypothesis 3 and 4

C. Chapter V will test hypothesis 5, 6,7 and 8

D. Chapter VI will test hypothesis 9, 10,11, and 12

E. **Chapter VII will be a general discussion of all of the results as they relate to the overall aims of this thesis**

2.4 REFERENCES

Alverdy,J.C. 1990. Effects of Glutamine-Supplemented Diets on Immunology of the Gut. Journal of Parenteral and Enteral Nutrition 14: 109S-113S.

Brown,G.D. and Gordon,S. 2001. Immune recognition. A new receptor for beta-glucans. Nature 413: 36-37.

Chen,J.T. and Hasumi,K. 1993. Activation of peritoneal macrophages in patients with gynecological malignancies by sizofiran and recombinant interferon-gamma. Biotherapy 6: 189-194.

Field,C.J., Thomson,C.A., Van Aerde,J.E., Parrott,A., Euler,A., Lien,E., and Clandinin,M.T. 2000. Lower proportion of CD45R0+ cells and deficient interleukin-10 production by formula-fed infants, compared with human-fed, is corrected with supplementation of long-chain polyunsaturated fatty acids. J. Pediatr. Gastroenterol. Nutr. 31: 291-299.

Kim,H.M., Han,S.B., Oh,G.T., Kim,Y.H., Hong,D.H., Hong,N.D., and Yoo,I.D. 1996. Stimulation of humoral and cell mediated immunity by polysaccharide from mushroom Phellinus linteus. International Journal of Immunopharmacology 5: 303-?

Nabuurs,M.J. 1998. Weaning piglets as a model for studying pathophysiology of diarrhea. Vet. Q. 20 Suppl 3: S42-S45.

Newsholme,E.A. and Calder,P.C. 1997. The proposed role of glutamine in some cells of the immune system and speculative consequences for the whole animal. Nutrition 13: 728-730.

Paniagua,M., Espinoza,F., Ringman,M., Reizenstein,E., Svennerholm,A.M., and Hallander,H. 1997. Analysis of incidence of infection with enterotoxigenic Escherichia coli in a prospective cohort study of infant diarrhea in Nicaragua. J. Clin. Microbiol. 35: 1404-1410.

Ruggeberg,J., Stalmach,E., Zubrod-Eichert,C., Wahn,V., and Schroten,H. 1997. Antimicrobial Functions of Human Monocytes Depend on Concentration of glutamine in vitro. Annals of Nutrition Metabolism 41: 371-375.

Saalmuller,A., Hirt,W., and Reddehase,M.J. 1989. Phenotypic discrimination between thymic and extrathymic CD4-CD8- and CD4+CD8+ porcine T lymphocytes. Eur. J. Immunol. 19: 2011-2016.

Shipley,S.G. 1996. Glutamine in Total Parenteral Nutrition. Nutrition Today 31: 74-77.

Sinkora,J., Rehakova,Z., Sinkora,M., Cukrowska,B., and Tlaskalova-Hogenova,H. 2002. Early development of immune system in pigs. Vet. Immunol. Immunopathol. 87: 301-306.

Smith,R.J. 1990. Glutamine Metabolism and Its Physiological Importance. Journal of Parenteral and Enteral Nutrition 14: 40S-44S.

Tayeh,M.A. and Marletta,M.A. 1989. Macrophage oxidation of L-arginine to nitric oxide, nitrite, and nitrate. Tetrahydrobiopterin is required as a cofactor. J. Biol. Chem. 264: 19654-19658.

Williams,D.L. and Di Luzio,N.R. 1979. Glucan induced modification of experimental Staphylococcus aureus infection in normal, leukemic and immunosuppressed mice. Adv. Exp. Med. Biol. 121: 291-306.

Willment,J.A., Gordon,S., and Brown,G.D. 2001. Characterization of the human β-blucan receptor and its alternatively spliced isoforms. J Biol Chem 276: 43818-43823.

Wu,G., Meier,S.A., and Knabe,D.A. 1996. Dietary glutamine supplementation prevents jejunal atrophy in weaned pigs. J. Nutr. 126: 2578-2584.

Zhang,K. and Petty,H.R. 1994. Influence of polysaccharides on neutrophil function: specific antagonists suggest a model for cooperative saccharide-associated inhibition of immune complex-triggered superoxide production. J. Cell Biochem. 56: 225-235.

Chapter 3 – DEVELOPMENT OF AN ETEC SURGICAL MODEL IN YOUNG PIGLETS

3.1 INTRODUCTION

ETEC Models of Infection

To test potential preventative therapies and nutritional interventions, an animal model of ETEC is required. The development of a reproducible orally-induced model in the piglet has been problematic (Sarmiento et al. 1988; Nabuurs 1998). Contributing to this are the observations that the age of the piglet influences susceptibility to infection: neonatal piglets are susceptible to strains of ETEC that produce 987P or K99 pili. ETEC that produce K88 pili are commonly associated with diarrheal disease in both neonatal and newly weaned piglets (Dean et al. 1989). Additionally, K88+ bacteria do not adhere to the brush borders of all pigs (Sellwood 1979) and the amount of potential glycoprotein or glycolipid *E. coli* receptors in gut change with age (Conway et al. 1990).

Despite the use of ETEC-sensitive piglet strains, gavaging animals with K88+ ETEC strains does not consistently produce diarrheal infection (Sarmiento et al. 1988; Nabuurs 1998). Modification of environmental factors such as a reduction in environmental temperature (cold stress), gastric acid neutralization, abrupt weaning and removal of lacteal immunity, and brief exposure to post-weaning dietary antigens before weaning have been attempted, with limited success to increase the probability of obtaining a reproducible model (Sarmiento et al. 1988).

In vitro and *in vivo* Surgical Models

Various techniques have been developed to study the interactions of microorganisms and the gut-associated lymphoid tissue (GALT) *in vitro*. For example, excised intestinal tissue maintained in culture medium has been used in some studies (Girardeau et al. 1980; Conway et al. 1990), but tissue viability is poor (Jepson & Clark 1998). Fixed tissue has been utilized to examine bacterial adhesion (Snodgrass et al. 1981; Laux et al. 1984), but this does not represent *in vivo* conditions and modifications of bacterial receptors can occur during tissue preparation (Jepson & Clark 1998). The gut loop method offers the advantage that inoculation conditions and timing are more readily controlled, optimizing the likelihood of observing initial cellular interactions (Jepson & Clark 1998). Additionally, including a control loop within each animal can control for inter-animal differences.

In vivo physiological response to surgery and anesthesia

Surgery elicits profound physiological changes including hormonal, metabolic, haematological and immunological responses (reviewed in (Hall & Ali 1998), the magnitude of which are proportional to the invasiveness of surgery. The immunological responses will be described in detail later in this chapter. It is also necessary to describe some other responses that must be considered with surgical models. The concentrations of several hormones have been shown to be affected by surgical stress, including prolactin, cortisol, adrenocorticotropic hormone, growth hormone, and β-endorphin (Guieu et al. 1998; Castejon-Casado et al. 2001). The gastrointestinal tract is sensitive to surgical stress (reviewed by (Anup & Baslasubramanian 2000) and alterations in intestinal permeability have been reported following surgery (Roumen et al. 1993). While the cellular and subcellular changes that occur in the intestinal tract after surgical stress are not well documented, there are reports of increased production of ROI, functional changes in mitochondria, and increased proteolysis which would all alter the integrity of the gastrointestinal mucosa (Anup & Baslasubramanian 2000).

Effect of surgery and aneasthesia on immune function

When using an in situ model, the influence of the anesthetic and surgical procedure must be considered. Many functions of the immune system are altered after anaesthesia and surgery (Helmy et al. 1999), possibly explaining transient postoperative immunosuppression (Markovic & Murasko 1993) and contributing to morbidity and mortality from infection (Helmy et al. 1999). The principal immunological change after major surgery is decreased cell-mediated immunity, likely mediated by an impaired NK cell response T helper (Th1) response, resulting in preferential Th2-type responses (Helmy et al. 1999). Other immune changes reported following surgery/anesthesia include reduced total peripheral blood lymphocyte (PBL) counts (affecting both T lymphocyte (T cell) and B lymphocyte (B cell) numbers), decreased responses to mitogens (ConA) and microbial antigens, diminished delayed hypersensitivity reactions and decreased mixed lymphocyte responses (Markovic & Murasko 1993; Markovic et al. 1993; Lahteenmaki et al. 1998). Following surgery, enhanced antibody responses have also been documented (Markovic & Murasko 1993; Markovic et al. 1993; Lahteenmaki et al. 1998). This appears to accompany a decrease in serum immunoglobulin concentrations contributed to by haemodilution and the loss of protein into extravasal tissues (Lahteenmaki et al. 1998). Cytokines affected by the surgical process include Interleukin (IL)-2, whose production has been shown to significantly decrease after initiation of anaesthesia (Helmy et al. 1999). However, surgery is reported to result in increases in pro-inflammatory IL-6 (Helmy et al. 1999), TNF-α (Hall & Ali 1998; Helmy et al. 1999) and IL-1 (Hall & Ali 1998; Helmy et al. 1999; Bozkurt et al. 2000) and anti-inflammatory IL-4 (Helmy et al. 1999) cytokines in plasma. Neutrophil and macrophage functions reported to be enhanced with surgery include: increased neutrophil influx to sites of inflammation (Kotani et al. 1999), increased expression of chemoattractants (MIP-2) (Kotani et al. 1999), and increased microbicidal activity (adhesion and production superoxide ions) (Mobert et al. 1999).

The systemic effect of inoculating E. coli into intestinal loops

The gut loop model has been used previously for the isolation of intestinal loops in young piglets (Dugan & McBurney 1995; Adegoke et al. 1999a; Adegoke et al. 1999a; Adegoke et al. 2003). With the exception of a relatively minor effect of surgery and anesthesia on plasma amino acid levels (small increase in alanine, glycine and valine) (Adegoke et al. 1999a), few systemic effects have been reported. The effect of this procedure on immune function has not been studied.

The overall goal of this study was to develop a working, reproducible model of ETEC infection. The first approach was to establish a clinical model of ETEC infection. It was hypothesized that oral inoculation with live ETEC to weaned piglets would result in reproducible clinical symptoms of mild infection.

3.2 ETEC INFECTION TRIALS (Objective 1)

Part I – Oral inoculation of newly weaned piglets with 3 doses of ETEC

Materials and Methods

Preliminary trials were conducted in newly weaned piglets (2 Trials, n=9 & n=10). A variety of factors that influence infection were considered and manipulated in these trials, including ETEC stains, confirming the expression of K88 antigen on these stains, *E. coli* growing conditions (media constituents and consistency, temperature, shaking vs. still growth), bacterial concentration and inoculation procedures. All piglets used in accomplishing this objective were obtained from a local commercial swine herd (Shooter's Hill Livestock, Alberta). The initial experiment was conducted (August 1999) in piglets (n=3 per group) orally inoculated with bacteria (10^9 CFU/ml, 10^{10} CFU/ml or 10^{11} CFU/ml, 5 mls/day) with different frequencies (10^9, 10^{10} administered daily for 5 days, 10^{11} administered daily for 3 days). Cultures of ETEC strain E 681 (obtained from Microbiology Laboratory, Dept. of Agricultural Food and Nutritional Science, University of Alberta, Edmonton) were grown at 37^0C on solid agar Tryptic Soy Broth (TSB) plates to facilitate the ability of bacteria to express fimbriae (K88) (Thorns et al. 1989). Cultures were prepared (by post-doctoral fellow A. Gaydamaka) and with the assistance of the microbiology lab, the concentration determined by measuring absorbance and performing plate counts at specific time intervals. These cultures were diluted in sterile saline to the desired concentration. ETEC was administered by insertion of tubing into the stomach and syringe injection. The exact length necessary to reach the stomach was predetermined and marked on the tubing. To neutralize gastric acidity, sodium bicarbonate (1.2% w/v) was combined with the bacterial inoculum immediately prior to administration to piglets. The incidence and severity of diarrhea were recorded on a daily basis. Three blood samples were collected from each piglet (by Vena Cava puncture) in 10 ml Vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ) containing sodium heparin, which were shaken gently and stored on ice. The first sample was taken the day prior to ETEC inoculation, the second sample collected 72 hrs post-infection and the third sample collected 10 days post-infection at the conclusion of the trial.

Immunofluorescence

Lymphocyte subsets of freshly isolated immune cells in blood were identified by indirect immunofluorescence assay, as previously described (Field 1996). The monoclonal antibodies (mAb) combinations used in these studies is provided in the Appendix (Table 2). Non-sterile 96-well V-bottom microtiter plates (Fisher Scientific) were preconditioned with Phosphate Buffered Saline (PBS) containing Fetal Calf Serum (FCS) (40 v/L) at room temperature for at least 20 minutes. For blood cell phenotyping, 125 *u*l of whole blood was added to each well, followed by the addition of 200 *u*l of lysis buffer. This suspension was incubated 5-10 min. at 37°C, the plate was spun at 200 x g for 5 min. (Jouan Centrifuge), liquid was discarded, and the plate was vortexed to loosen pellets. These cells were washed with 4% v/v FCS in PBS (40 ml/L) to remove all media and lysis buffer. Plates were then spun at 200 x g (2 min), the liquid was discarded and pellet was vortexed. For indirect single-label (one colour) phenotype analysis, cells were then incubated for 30 min. at 4^0C with an aliquot of the primary antibody, washed three times in 200 uL of PBS containing FCS (40 ml/L), and incubated another 30 min. at 4^0C with 50 *u*L of a 1:300 dilution of fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Cedarlane, Hornby, Ont) that has no cross reaction to swine IgG. To determine background fluorescence due to non-specific binding of FITC, samples were incubated for 30 min. at 4^0C with FITC alone. For double-label (two colour) immunofluorescence, the phenotypic antibody was incubated with FITC as described above. Following FITC incubation and washing, an aliquot of the secondary antibody was added to each well and incubated for 30 min. at 4^0C. Cells were then washed three times (as described above) prior to incubation for 30 min at 4^0C with 10 uL of a 1:25 dilution of phycoerythrin (PE)-conjugated goat anti-mouse IgG (Cedarlane Laboratories Ltd.). As with FITC background, samples were incubated for 30 min. at 4^0C with PE alone to determine background fluorescence due to non-specific binding. Finally, the cells were washed 2 times (as described above), fixed in 200 uL of PBS containing paraformaldehyde (10 g/L; Anachemia Science, Montreal, Quebec), and relative fluorescence intensities for each antibody determined by flow cytometry (FACScan, Becton Dickinson, Sunnyvale, CA). Unwanted events (dead cells and debris) were detected on the basis of forward scatter and side scatter and were excluded from subsequent phenotype analyses by electronic gating of the viable splenocyte population. Ten-thousand viable events were collected in list mode and all subsequent immunofluorescence analyses were performed on only these cells using Lysis II software (Becton Dickinson). The resulting percentages were corrected for background fluorescence using the analysis of cell incubated with FITC or PE alone.

Neutrophil Function

Whole blood was used to assess neutrophil function (Vowells et al. 1995). Briefly, 4 mls of warm lysis buffer [8.30 g/L NH_4Cl, 1.68 g/L $NaHCO_3$, 2 mls 0.5M ethylenediaminetetraacetic acid (EDTA, pH=8.0), dissolved in ddH_2O] were added to 400 *u*l whole blood aliquots and tubes were shaken 5 mins. in 37°C water bath. Cells were pelleted by spinning at 450 x g (5 mins.). Supernatant was discarded and tubes were blotted by wicking adherent fluid from the top of each tube. Each pellet was then washed and re-suspended with 4 mls wash buffer (1 g/L BSA, 2 mls 0.5 M EDTA, dissolved in ddH_20). The spin was repeated, supernatant discarded, and tubes blotted. Each pellet was then re-suspended with 400 *u*l wash buffer, to which 1.8 *u*l Dihydrorhodamine (DHR 123, Molecular Probes, Eugene, OR; 25 ul aliquots stored at -70°C, dissolved in Dimethyl sulphoxide (DMSO at 29 mM)) was added and incubated 5 min. in the 37°C waterbath. A 100 *u*l sample was taken from each tube and placed immediately on ice (Time = 0). Polymorphonuclear neutrophils (neutrophils) were stimulated with 100 *u*l of phorbol myristate acetate (PMA, Sigma; 10 *u*l aliquots dissolved in DMSO at 2 *ug*/*u*l, diluted with 990 *u*l wash buffer) mitogen and returned to the water bath. Successive samples (100 *u*l) were taken and placed directly on ice at 5 and 10 min. At 15 minutes the assay was stopped by removing the remaining tubes and placing directly on ice. Neutrophil oxidative burst, granularity, and size were assessed immediately by flow cytometry (FACScan, Becton Dickinson). Neutrophils at all time points were acquired (10,000 gated events) using Neuts Aquan Acquisition software (Becton Dickinson; SSC=300, FL1=329). During analysis the gate was adjusted to include a uniform population and the mean fluorescence (oxidative burst), forward scatter (surface area) and side scatter (granularity) measures were transferred to an Excel spreadsheet (Microsoft) for statistical analysis.

Results

Only piglets receiving the medium to high doses of ETEC showed any signs of infection (Table 1). Two piglets were euthanized before the conclusion of the trial; one animal receiving 10^{11} CFU/ml (5mls/day) because of severe diarrhea and one animal receiving 10^{9} CFU/ml (5mls/day) because of extreme weakness and low feed consumption. Characterization of immune cell phenotypes revealed no significant differences in expression of T cell antigens (Figure 1). Calculation of the CD4/CD8 ratio revealed a gradual increase (0.5 ± 0.1 in 10^{9}CFU/ml, 0.7 ± 0.05 in 10^{10}CFU/ml, and 0.9 ± 0.1 in 10^{11}CFU/ml) with increasing ETEC exposure that was not significant. However, the proportion of monocytes was significantly increased in piglets inoculated with 10^{11} CFU/ml and 10^{10} CFU/ml 10 days post-infection (Figure 1). The proportion of B cells was significantly increased in piglets inoculated with 10^{10}CFU/ml compared to piglets inoculated with 10^{9} CFU/ml (Figure 1). Neutrophil oxidative burst fold increase (0 to 5 and 0 to 10 min) was significantly increased 72 hrs post-infection in piglets inoculated with 10^{11} CFU/ml ETEC (Figure 2).

Higher doses of ETEC (10^{10} and 10^{11} CFU/ml) were shown to have some influence on piglet diarrhea score. One animal (high dose) developed diarrhea that was considered severe. One animal receiving the medium ETEC dose showed transient clinical signs of diarrhea, but recovered quickly. The first animal (from low dose group) was euthanized because of extreme weakness that could not definitely be attributed to the exposure to ETEC. Although only two out of nine animals were observed to have diarrhea incidence, these results suggested that the medium and high doses of ETEC might be appropriate for further study.

Discussion

Compared to piglets inoculated with the low dose of ETEC, there was a significantly increased proportion of monocytes in piglets inoculated with medium and high doses of ETEC. There was also an increased proportion of B cells in peripheral blood of piglets in the medium-dose group compared to the low dose group. Increases in these two immune populations suggest that T cell number would also be altered, but there were no dose-related changes in any T cell phenotypes. However, although there were no significant differences in CD4/CD8 ratio, the trend to increase with increasing ETEC suggests that the immune system of these animals had been challenged. The small group size likely contributed to the lack of effect, which may be more visible with larger group sizes. Previously, altered T cell function (increased CD4/CD8 ratio and decreased CD8+ cell numbers) was demonstrated in sheep suffering from bacteria-induced diarrhea (Larsen et al. 1999).

Results of this infection trial suggested the higher ETEC doses induced some clinical and immune indications of infection. However, the ability to produce these effects in all animals treated with ETEC was not consistent. The small number of animals treated in each group and high intrapiglet variability may have contributed to this. Therefore, to determine the reproducibility of the clinical findings from the study, a subsequent infection trial was performed using a larger number of piglets.

Part II - Oral inoculation of newly weaned piglets with 2 high doses of ETEC

Materials and Methods

Piglets (n=5 per group) were administered the higher doses of bacteria used in the previous experiment (10^{10} & 10^{11} CFU/ml, 5 mls/day)) over a longer period of time (5 d). To reduce the labor of growing and harvest of ETEC from solid plates, TSB broth was used as the growth medium. Cultures of ETEC strain E 681 were grown at 37^0C in a shaking incubator. ETEC cultures were diluted to the desired concentration in sterile saline solution. ETEC was administered by insertion of tubing into the stomach and syringe injection. The exact length necessary to reach the stomach was pre-determined and marked on the tubing. The incidence and severity of diarrhea was recorded on a daily basis by the same researcher. As described in Part I, PBL were isolated for identification of lymphocyte subsets by indirect immunofluorescence and neutrophil function was assessed on whole blood samples.

Results

Piglets inoculated with either 10^{10} CFU/ml or 10^{11} CFU/ml developed no clinical signs of diarrhea (Table 2). Two piglets were euthanized before the conclusion of the trial because of extreme weakness and low feed consumption. Subsequent necropsy showed that both animals showed signs of bronchopneumonia. Compared to immune cell phenotypes of PBL isolated prior to ETEC inoculation, there was a significant increase in the proportion of CD4+ cells 10 days post-ETEC inoculation (Figure 3). However, at 10 days post-inoculation, the proportion of CD8+, CD45RA+, and CD25+ cells were all significantly decreased compared to immune cells obtained prior to ETEC treatment (Figure 3). Furthermore, in piglets receiving the higher ETEC dose, the CD4/CD8 ratio was significantly increased ($P<0.005$) at 10 days post infection (1.5 ± 0.2) compared to ratios pre-infection (0.7 ± 0.1) or 72 hrs post-infection (0.8 ± 0.1). In piglets inoculated with 10^{11} CFU/ml ETEC, neutrophil oxidative burst (15 min) was significantly increased at 72 hrs- and 10 days post-infection compared to the oxidative burst of neutrophils obtained prior to ETEC inoculation (Figure 4).

Discussion

Based on results from the previous trial, the lower ETEC dose was dropped and a larger number of animals were used. In this subsequent trial, the highest ETEC dose influenced the proportion of different immune cell populations and neutrophil function. Neutrophil peak oxidative burst (15 min) was significantly increased both 72 h and 10 days post-ETEC inoculation, suggesting that neutrophil function was increased. Piglets inoculated with the higher ETEC dose had a significantly higher CD4/CD8 ratio that was a result of both a decrease in CD8+ cells and an increase in CD4+ cells. The increased expression of CD25 on T cells suggests that these cells have been activated by *in vivo* antigen exposure. This has been demonstrated previously with diarrhea infections in other species (Larsen et al. 1999). The decrease in CD45RA (antigen naïve phenotype) is also consistent with the early signs of infection because less antigen naïve cells indicates that more immune cells have been exposed to antigen.

Necropsy of two animals which were euthanized due to illness suggested that these animals had bronchopneumonia, most likely a result of bacteria accidentally being inoculated into the lungs. In the remaining piglets, despite the observed results on immune cell function that indicated a mild infection, no animals showed clinical signs of diarrhea. There were several potential explanations for this, including the possibilities that the piglets did not express the K88 binding sites on their epithelial cells, the strains of ETEC used in these studies were not highly pathogenic, or the conditions under which these strains were grown reduced their pathogenicity. It was concluded that the failure to clearly achieve clinical diarrhea, together with the labor intensiveness of the study and the number of animals that would be required to achieve sufficient power to attempt dietary intervention, continued refinement of this model was not warranted.

3.3 DEVELOPMENT OF SURGICAL MODEL (Objective 2)

It was hypothesized that an *in vivo* loop model would produce the early signs of ETEC infection. The gut loop model was used to examine the immediate immune and gut response to ETEC. It was understood that these measures would be influenced by the established effects of surgery and anesthetic on systemic immune function (Hall & Ali 1998), gut function (Anup & Baslasubramanian 2000) and more minor effect on plasma amino acids (Adegoke et al. 1999a). Therefore, before adopting this model, the following was required: to characterize the effects of the surgical procedure on systemic immune function and to ensure that inoculation of ETEC is sufficient to induce the early signs of infection in isolated loops.

Materials and Methods

A number of pilot surgeries (11 total) were performed in developing this model. This model was based on the principles of an *in situ* intestinal gut loop piglet model that was previously developed for assessing intestinal amino acid metabolism (Adegoke et al. 1999a). The minor modification to this method involved tying off both ends of the loops, resulting in a closed loop, instead of open loop, system. Prior to each surgery, *E. coli* cultures were prepared fresh from frozen stock. Upon consultation with Dr. M. Stiles (University of Alberta) and Dr. C. Gyles (University of Guelph), brain-heart infusion (BHI) media was used (instead of TSB). Fresh cultures of ETEC strain PDH08 (kindly provided by Dr. Carlton Gyles, University of Guelph, Guelph, ON, Canada) were prepared by transferring a small sample of ETEC frozen culture to 6 mls of BHI media (Oxoid LTD., Basingstoke, Hampshire, England) and growing for 24 hrs (in shaking incubator at 37°C). This ETEC culture was subsequently subcultured (24 hrs). The final culture was prepared by transfer of 2 mls of subcultured ETEC media to a 200ml flask with BHI media (Oxoid) and grown in a shaking incubator at 37^0C. A sample from this flask is taken (after approximately 12 hrs) and diluted to a final concentration of approximately 1×10^9 CFU/ml. The concentration of these cultures was determined as described previously in this chapter. The bacteria were re-suspended in sterile culture tubes with 30 mls PBS with an osmolarity (300 mOsm/L) similar to normal intestinal content (250-380 mOsmoles) (Adegoke et al. 1999b). Sterile tubes were transported in a biohazard container and kept warm until used in the surgical procedure.

A total of 11 piglets at approximately 35 days of age were obtained from the University of Alberta Swine Research Technology Centre and transported to the Metabolic Research Unit. Piglets received intramuscular injections of Torbugesic (0.2 mg/kg), Ketamine (11 mg/kg), Rompun (2.2 mg/kg), and Robinul (0.01 mg/kg). Surgeries were performed in a separate, closed surgery room and anaesthesia was maintained with 1.0-1.5% v/v halothane delivered with 3L/min oxygen. An electrical heating pad and an over-head infra red heating lamp was used to maintain body temperature, which was monitored frequently with rectal thermometer readings. Following anesthesia, the animal was placed on the surgical table and the incision area was scrubbed with antibiotic soap. A vertical incision was then made slightly below the sternum and extended to about 20 cm to open the abdominal cavity. Intestinal loops were then isolated and measured to approximately 10 cm in length. The first intestinal loop was located 15 cm from the ligament of Treitz, and each successive section was separated by 50 cm of intestine. The ties were inserted through the mesentery and tied loosely around each end. Before tying off the distal end of the loop, the segment was flushed with PBS, which was then smoothed through the segment. Loops not exposed to ETEC (non-ETEC) were inoculated with PBS solution containing no pathogen. Both the PBS and the bacterial treatments were administered with 30 ml syringes (Becton Dickinson) and 1 inch, 20 ½ guage needles (Becton Dickinson). The needle was inserted into the intestine, just before the first tie. Loops were filled with the entire solution or until they were fully distended. Each segment was then tied off with labeled pieces of braided umbilical tape (Baxter, Deerfield, IL) while trying to minimize any backflow. A total of four intestinal loops were isolated and 3 treatments were administered. Following an incubation period of approximately 4 hours, euthanasia was induced (cardiac injection of Pentobarbital Euthanyl – 2 ml/4.5 kg body weight (BW)), gut sections were removed and immersed in ice-cold PBS.

Sample Collection

Three blood samples were collected (by vena cava puncture) during the course of each surgery in 10 ml Vacutainero® tubes (Becton Dickinson, Franklin Lakes, NJ) containing sodium heparin, shaken mildly and stored on ice. These samples were used to monitor immune changes that occur due to the surgical process. The first sample was taken before the animal was exposed to anesthetic (pre-anesthetic), the second just previous to beginning the surgery (pre-surgery), and the third just prior to termination by euthanasia (post). The animal was kept anesthetized and temperature was monitored throughout the surgery. Treatment suspensions were incubated for up to four hours. In previous studies, neutrophil influx was not observed until 4 h after anesthesia and surgery (Kotani et al. 1999), which was assumed, but not measured, in our model. If this did occur, the use of a non-inoculated ETEC loop within each animal would ensure that the infiltration due to surgery was similar between loops. At this time, euthanasia was induced (Euthanyl 1mg/4kg) and final tissue samples were taken. Mesenteric lymph nodes (MLN) were excised adjacent to the distal ileum (10-20 cm prior to the ileocecal junction), transferred to 50 ml sterile culture tubes (Fisher Scientific, Pittsburgh, PA) containing ice-cold PBS and stored on ice. All samples were transported immediately back to the laboratory at the Department of Agricultural, Food and Nutritional Sciences for further processing and analysis.

Immune Cell Isolation

PBL were isolated by centrifuging blood samples at 750 x g for 10 mins to remove plasma. The samples were topped up with 1% (v/w) Bovine Serum Albumin (BSA) buffer to dilute and re-suspend the red cell pellet and buffy coat. Four mls of the cell suspension was then layered over 4 mls Histopaque 1077 (Signa, Oakville, ON) and spun for 30 min. at room temperature (450 x g, no brake). The lymphocyte band was then removed and washed with 10 mls buffer. This suspension was then topped off with buffer and spun at 450 x g for 10 min. at 4°C. Supernatant was discarded and the pellet was re-suspended with 2 mls complete culture media [CCM; RPMI 1640 (Invitrogen, Burlington, ON) supplemented with 5 % (v/v) heat-inactivated fetal calf serum (FCS; Invitrogen), 1% (v/v) antibiotic-antimycotic solution, glutamine (gln; 4 mmol/L; Invitrogen), HEPES (25 mmol/L; Invitrogen), and 2-mercaptoethanol (2.5 umol/L; Sigma Chemical, Oakville, ON)]. Immune cell viability and counts were performed by trypan blue exclusion (Sigma Chemical) and using a stage micrometer at 100x magnification.

Under sterile conditions, MLN were dissected by removing connective tissue. Cells were then pressed through 100 *u*m mesh nylon screen using the barrel of a sterile syringe and buffer (0.5% BSA in KRH). The resulting cell suspension was centrifuged at 200 x g (Jouan centrifuge model CR 422, Canberra Packard, Mississauga, ON) for 10 min at 4^{0}C to pellet cells. To remove excess RBCs, lysis buffer (78 mM NH_4Cl; 10 mM $NaHCO_3$; 0.1 mM EDTA) was added to each suspension and spun at 200 x g for 10 min. The supernatant was aspirated and immune cells were re-suspended in CCM.

Immune Measures

Blood lymphocytes function was assessed by culturing isolated cells and quantifying the incorporation of ^{3}H-thymidine measured in response to mitogen. The mitogen response assay was conducted in triplicate on microtiter plates, as previously described (Field 1996). Blood immune cells in complete culture media (CCM)(1 x 10^6 cells/ml) were incubated in 96 well plates (Fisher Scientific) for 48 h in a humidified atmosphere at 37^0C in the presence of 5% v/v CO_2. Unstimulated immune cells were compared to cells stimulated with the mitogens phytohemagglutinin (PHA; 25 *u*g/ml; Sigma), pokeweed mitogen (PWM; 55 *u*g/ml; Sigma) and lipopolysaccaride (LPS; 1 mg/ml; Sigma). Each well was pulsed with 1 *u*Ci/*u*l ^{3}H-thymidine (Amersham Life Sciences, Brue D'Urfe, Quebec) 18 h prior harvest and proliferative rate was estimated by incorporation of ^{3}H-thymidine. Immune cells were harvested (Filtermate Harvester, Canberra Packard) and plate counts were recorded (Top Count NXT, Microplate Scintillation and Luminescence Counter, Canberra Packard). Stimulation indices (SI) were calculated by dividing the rate of ^{3}H-thymidine incorporation of stimulated cells by the rate of ^{3}H-thymidine incorporation of unstimulated cells. Lymphocyte subsets of freshly isolated immune cells in blood were identified by indirect immunofluorescence as previously described (Section 3.2). Whole blood samples were used to assess neutrophil function as previously described (Section 3.2).

Ussing Chamber Measures

A small section from each intestinal loop was removed, filled with PBS, and transported in 50 ml Falcon tubes filled with ice-cold PBS to preserve intestinal tissue. Before being mounted in Ussing Chambers, the serosa was removed from each segment. To maintain tissue viability, all tissue segments were mounted in the chambers within 1 hour of the piglet's death and, once mounted, each section was bathed in Ringer's solution with continuously circulating O_2 (95% v/v) and CO_2 (5% v/v). The tissue was continuously short-circuited (Isc) with automatic voltage clamps (model DVC 1000, World Precision Instruments, New Haven, CT), which compensated for the fluid resistance. Current was introduced to half-cells through agar (Ag-AgCl) electrodes which were connected to the chambers via agar bridges. Potential difference (PD) across the tissue was measured by calomel voltage electrodes (2% w/v purified agar dissolved in 3M KCl), positioned near the surface of the tissue. Intestinal Mannitol permeability (ManP) was assessed by adding ^{3}H-mannitol (1mCi, PerkinElmer, Boston, MA) to the reservoir exposed to the mucosal side of the tissue. Each tissue was given a 15 min. stabilization period, during which time 2 initial samples were taken from this same mucosal reservoir. Following this stabilization period, the tissue's permeability to mannitol was measured by sampling for its appearance on the opposite (Serosal) side at 5 min intervals. Counts on each sample were performed with a liquid scintillation counter (betaray scintillation, Beckman Coulter, Fullerton, CA) and the average of three values of mannitol flux was used as the ManP of each tissue. Prior to each sample period, electrical parameters (PD & Isc) were recorded from the voltage clamp, leading from the current and voltage electrodes connected to the chamber near the tissue segment. From each of these values, conductance (G) was calculated from the open circuit PD and Isc using Ohm's law. Finally, at the end of the sampling period, tissue viability was assessed by adding the adenyl cyclase activator forskolin (Fors; 10 ul, Sigma) to the serosal reservoir. The Fors score was recorded as the peak changes in Isc readings.

Results

The anesthesia and the surgical process did not significantly influence peripheral blood phenotype (n=8), after a general anesthetic or when 2 loops in a piglet were incubated with ETEC for 4 hours (Figure 5). Similarly, the neutrophil oxidative burst (n=5) was not significantly affected by the anesthetic nor the presence of *E. coli* in the intestinal loop (Figure 6). This suggests that the physiological effect of inoculating loops with ETEC does not significantly affect these representative estimates of systemic immune function during a 4 hour in situ incubation with ETEC. After the surgical process with ETEC challenge in two of the four loops, cells isolated from peripheral blood and incubated in the absence of mitogen did not show changes in their ability to incorporate ^{3}H-thymidine (Figure 7). Following stimulation with mitogen, MLN isolated following the surgical procedure responded well (defined by a robust rate of ^{3}H-thymidine uptake) (Figure 8). However, since MLN could not be sampled prior to the surgical process, one can only conclude that there appeared to be no outright signs of immunosuppression from the surgical process in this GALT tissue. The surgical procedure did not influence the ability of PBL to incorporate ^{3}H-thymidine following incubated with mitogen (Figure 9). Differences in ManP and G (as assessed in Ussing chambers) were observed between the ETEC inoculated, as compared to the placebo infused loops from the same animal (Table 3). This suggests that the incubation period altered tight junction integrity and thus initiated the early stages of ETEC infection. A photograph of the surgical procedure is shown in Figure 10.

Discussion

In developing the surgical model, MLN were collected from the distal ileum to perform functional assays on isolated immune cells. Although not described here, in future studies PP lymphocyte populations and function were also characterized. In order to maximize the number of isolated immune cells, it was decided to collect cells from the continuous ileal PP, which may extend for up to 2.5 m along the terminal ileum, as opposed to the more numerous jejunal PPs, which are comparatively small (Stokes et al. 1996). The ileal PP is also considered a primary B-cell organ because it contains more than 90% B cells (Andersen et al. 1999), whereas the jejunal patches contain roughly equal percentages of B and T cells (Pabst et al. 1988). After making this decision, it was logical to collect MLN adjacent to the distal ileum. While much remains to be determined regarding the immune populations in the MLN, it would be reasonable to predict that immune cells located in the distal region of the intestine would contain cells that have migrated from the ileal PP.

Exposure of enterocytes to ETEC influences several physiologic parameters. In this study, the early stages of infection were assessed by Ussing chamber measures. Observed differences in intestinal permeability of isolated loops following ETEC inoculation suggests that a 4 hr incubation period was sufficient to bring about the early stages of infection. As it has been established that surgery influences the permeability of the gut, it is logical to study some of the animals prior to initiating the ETEC model. Although anesthetic is still used and there is surgery to remove the segment, exposure to both of these potential confounding variable is significantly reduced. It was decided that this would be included in one of the later experiments. To eliminate the influence of anesthesia and surgery in studies examining the dietary effects on peripheral mononuclear cell function, blood was collected from animals prior to being subjected to this *in situ* model.

In conclusion, the *in situ* model offers the advantages of an *in vivo* system in that the gut response can be assessed, within the same animal, the function of various parameters of GALT can be measured and utilization of a smaller number of animals is possible, which also minimizes intrapiglet variation.

3.4 DEVELOPMENT OF ETEC ADHESION ASSAY (Objective 3)

Adhesion of ETEC to piglet enterocytes has been previously described (Girardeau 1980; Francis et al. 1998). However these qualitative methods involve exposure of enterocytes or gut sections to ETEC and microscopic observation. The purpose of these studies was to develop an *in vitro* binding assay to assess ETEC binding to isolated enterocytes.

Materials and Methods

Methods developed by Clarke *et al.* (Clarke & Morton 2000) were used in development of this assay, which are shown in the Appendix (Figure 1). However, when samples were taken to be analyzed by flow cytometry, the purity of the mixture and the remnants from the intestinal mucosa, particularly mucus, dramatically affected fluorescence measurements. To further purify the enterocyte culture, intestinal segments were washed according to the methods of Madsen et al. (Madsen et al. 1995). The chemical phenylmethylsulfonyl fluoride (PMSF; 0.2 mM; Sigma) was used as a protease inhibitor and dithiothreitol (DTT; 0.5 mM; Sigma) was used to dissolve mucus. To remove mucosal debris, enterocytes collected from segment washes were centrifuged on a PERCOLL gradient (30-70% w/v). This gradient centrifugation forms an upper epithelial cell layer that was then collected for adhesion studies. A method developed by Fan et al. (Fan et al. 2001) was used to isolate viable enterocytes that were virtually mucus-free. During the isolation washes, cells were oxygenated to maintain viability. Isolated mucosal cells were combined with FITC-labeled ETEC according to the methods developed by Clarke et al. (Clarke & Morton 2000) in which bacteria and enterocytes were combined for more than 45 min. Unlike these methods however, adhesion was quantified by flow cytometry (FACSCAN, University of Alberta Heritage Building).

Results

Results showed that obtaining enterocytes with the compounds listed and after spinning on a PERCOLL gradient was able to remove a great deal of the mucus present. This also resulted in a definite improvement in the purity of enterocyte isolations, which is illustrated in Figure 11. However, flow cytometry data seemed to indicate that binding of approximately 65-90% of FITC-labelled ETEC was occurring, depending on the sample being analyzed (Figure 12). There were also concerns that the large amount of cellular debris detected by flow cytometry may be indicative that the lengthy isolation procedures may have influenced the viability of isolated enterocytes.

Discussion

ETEC induce diarrhea in swine during the immediate neonatal period {<6 days of age}, and immediately after weaning (3 to 9 weeks old) (Dean et al. 1989). Therefore, it was expected that with age, binding of ETEC to mucosal cells would decrease. Analysis of the ETEC binding assay showed that an effect of age could not be shown by this method. The maximal binding that occurred following the incubation period did not allow for any differences to be found. Therefore, a new method for quantifying adhesion of K88 ETEC to mucosal cells was deemed necessary.

3.5 SUMMARY AND CONCLUSIONS

It was hypothesized that oral inoculation with live ETEC to weaned piglets would result in reproducible clinical symptoms of infection. Several preliminary trials were conducted in which a variety of factors that influence infection were considered and manipulated. However, the results of these trials demonstrated a great deal of variability in terms of severity of illness and immune response. Therefore, a large number of animals would be needed to test a dietary hypothesis. This led to the hypothesis that an *in vivo* loop model would produce the early signs of ETEC infection. Observed differences in intestinal permeability of isolated loops following ETEC inoculation supports the objectives of studying the early pathophysiological response of the gut to ETEC infection. This *in situ* model developed in this chapter was used in testing the main hypothesis of this thesis.

Table 1. Incidence and Severity of Diarrhea in newly weaned piglets. Piglets were inoculated with low (10^9 CFU/ml) and medium doses (10^{10} CFU/ml) 5 times (16/08/99-20/08/99) and with the high dose (10^{11} CFU/ml) 3 times (16/08/99-18/99).

		17/08/99	18/08/99	19/08/99	20/08/99	21/08/99	22/08/99	23/08/99	24/08/99	25/08/99	26/08/99
Low	Pig 1	0[1]	0	0							
	Pig 2	0	0	0	0	0	0	0	0	0	0
	Pig 3	0	0	0	0	0	0	0	0	0	0
Medium	Pig 4	0	0	0	0	0	0	0	0	0	0
	Pig 5	0	0	1 - 1[3]	0	0	0	0	0	0	0
	Pig 6	0	0	0	0	0	0	0	0	0	0
High	Pig 7	1 - 3	1 - 3	1 – 3							
	Pig 8	1 - 1	1 - 1	0	0	0	0	0	0	0	0
	Pig 9	1 - 1	1 - 1	0	0	0	0	0	0	0	0

[1] incidence: 0-none, 1-occurrence

[3] diarrhea severity scale: 1-slight diarrhea, 2-diarrhea, 3-severe diarrhea

Table 2. Incidence and Severity of Diarrhea in newly weaned piglet inoculated with medium (10^{10} CFU/ml) and high doses (10^{11} CFU/ml) of ETEC (20/09/99-24/08/99).

		21/09/99	22/09/99	23/09/99	24/09/99	25/09/99	26/09/99	27/09/99	28/09/99	29/09/99	30/09/99
Medium	Pig 1	0	0	0	0	0	0	0	0	0	0
	Pig 2	0	0	0	0	0	0	0	0	0	0
	Pig 3	0	0	0	0	0	0	0	0	0	0
	Pig 4	0	0	0	0	0	0	0	0	0	0
	Pig 5	0	0	0	0[2]						
High	Pig 6	0	0	0	0	0	0	0	0	0	0
	Pig 7	0	0	0	0	0	0	0	0	0	0
	Pig 8	0	0	0	0	0	0	0	0	0	0
	Pig 9	0	0	0	0	0	0	0	0	0	0
	Pig 10	0	0	0	0	0	0	0[2]			

[1] incidence: 0-none, 1-occurrence

Table 3. Effects of infusion of *E. coli in situ* on measures of intestinal permeability of isolated segments. Note: These results represent isolated segments in USSING chambers from gut loops infused with different treatments. The same *E. coli* solution was infused into 3 loops (replicates 1, 2 & 3). All experiments were done in the same 30 day old piglet. Interpretation of these preliminary results suggest that infusing a gut loop with *E.coli* had an effect on both increasing G and ManP (interpreted as increased permeability) and decreasing Isc response to Fors (interpreted as decreased stimulation of Cyclic $3^1,5^1$-adenosine monophosphate (cAMP) and decreased Chloride secretion).

Treatment in the in situ loop	G (mS/cm^2)*	ManP (pmol/cm^2)**	Fors (Δ Isc)***
Non-ETEC (vehicle/PSB)	14	10.2	61.1
E.coli solution replicate 1	18.7	14.2	53.5
E.coli solution replicate 2	18.4	17.9	14.3
E.coli solution replicate 3	18.7	18.2	35

* - units as milliSiemens/centimeter squared

** - units as picomoles/centimeter squared

*** - units as change in short-circuit current (uA/cm2)

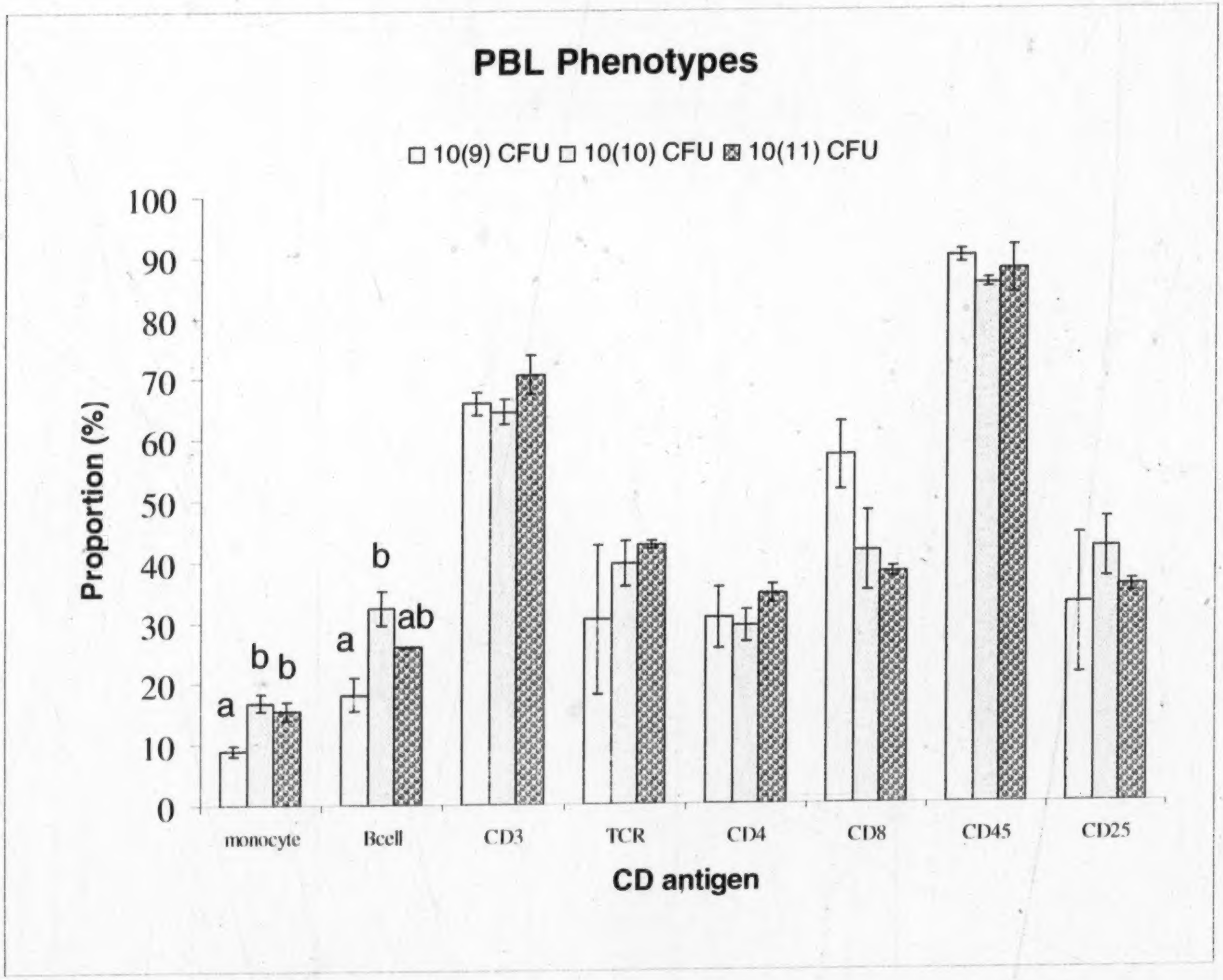


Figure 1. PBL Phenotypes of newly weaned piglets inoculated with 3 doses of ETEC.

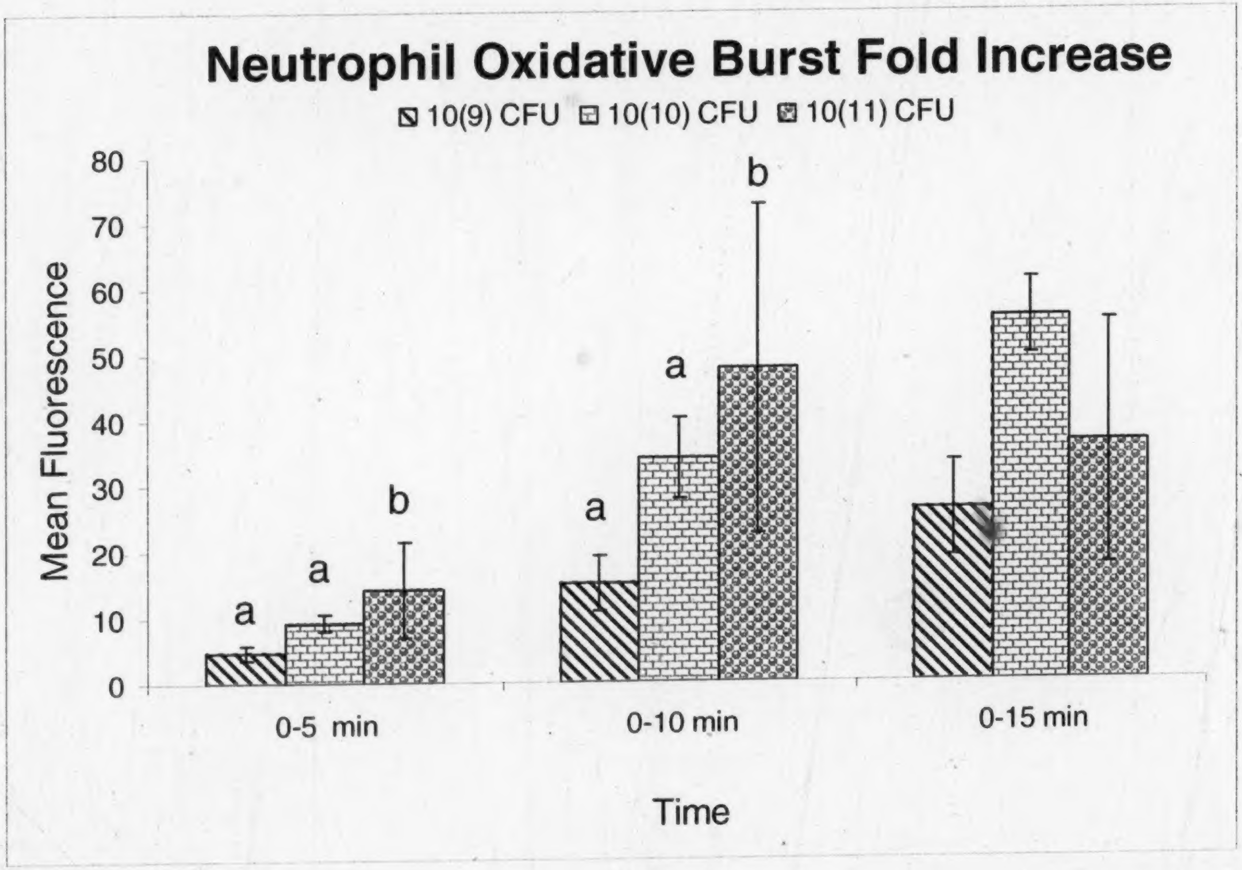


Figure 2. Fold Increase of neutrophil oxidative burst obtained from piglets 72 hours post-infection. Piglets were grouped according to dose of bacteria inoculated (10^9 CFU/ml, 10^{10} CFU/ml, 10^{11} CFU/ml)

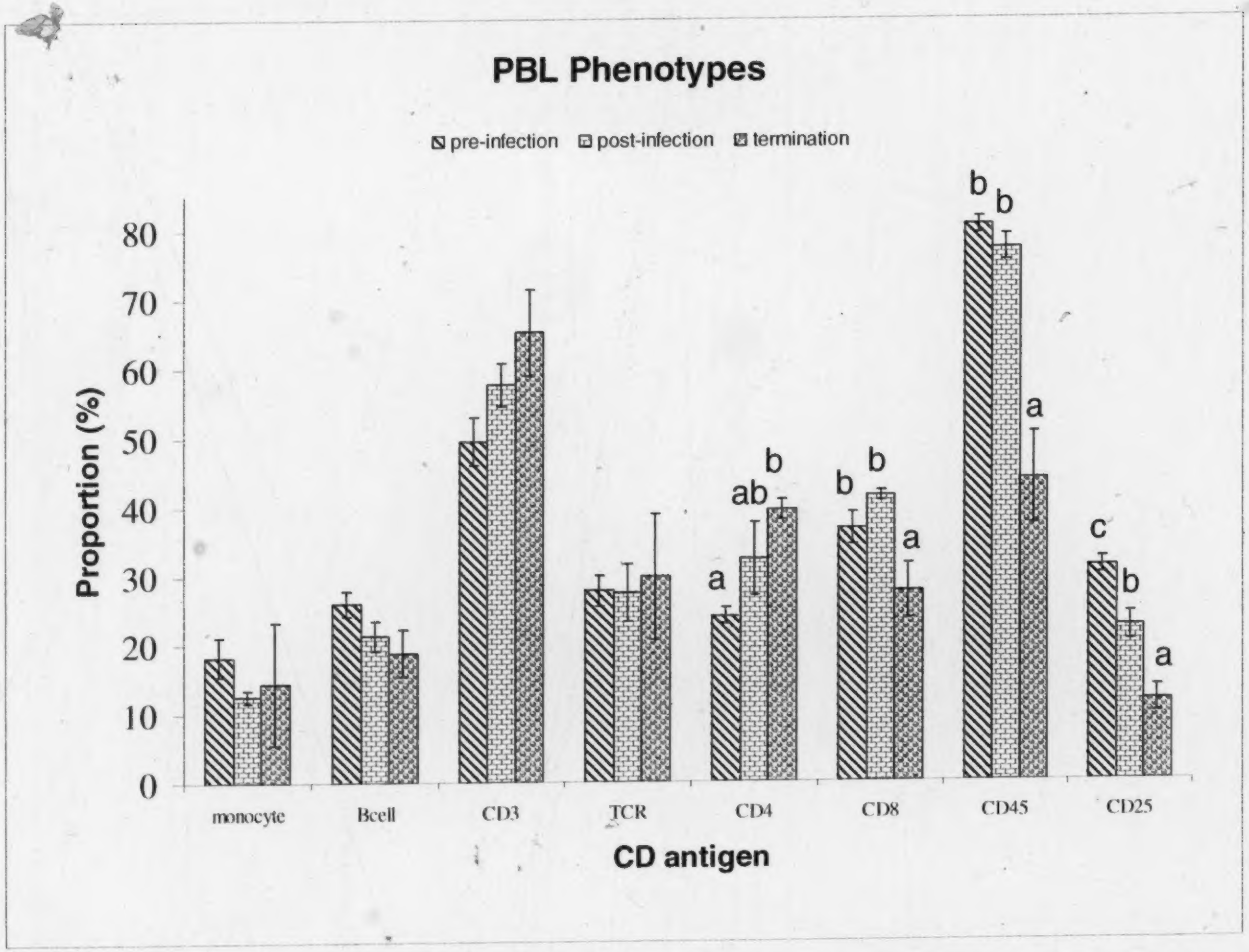


Figure 3. PBL phenotypes of piglets inoculated with ETEC (10^{11} CFUI/ml) and taken prior to inoculation (pre-infection), 72 hours following the first inoculation (post-infection) and 10 days following the initial inoculation (termination).

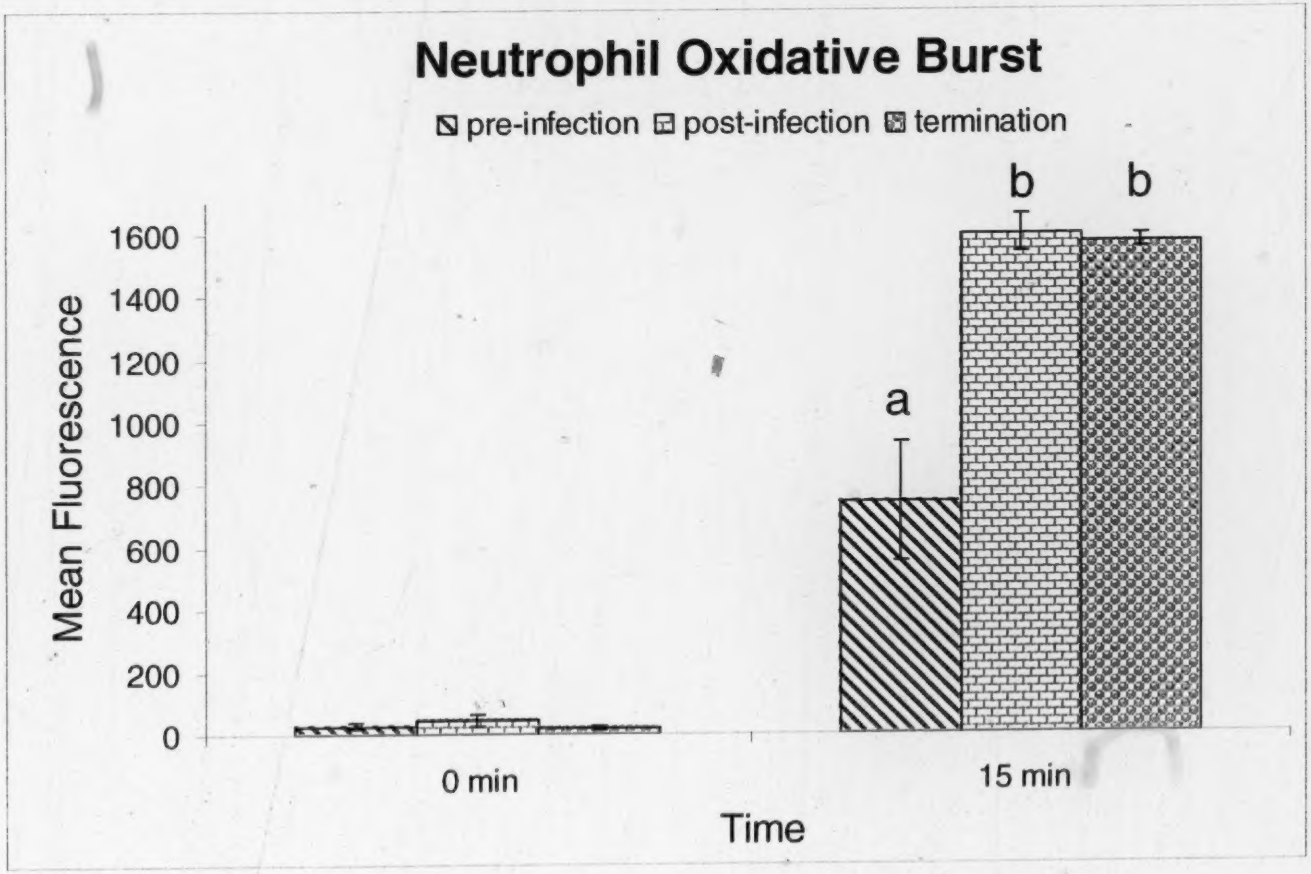


Figure 4. Piglet neutrophil oxidative burst. Whole blood samples were taken from piglets the day before the first inoculation (pre-infection), 72 hours following the first inoculation (post-infection), and 10 days following the first inoculation (termination).

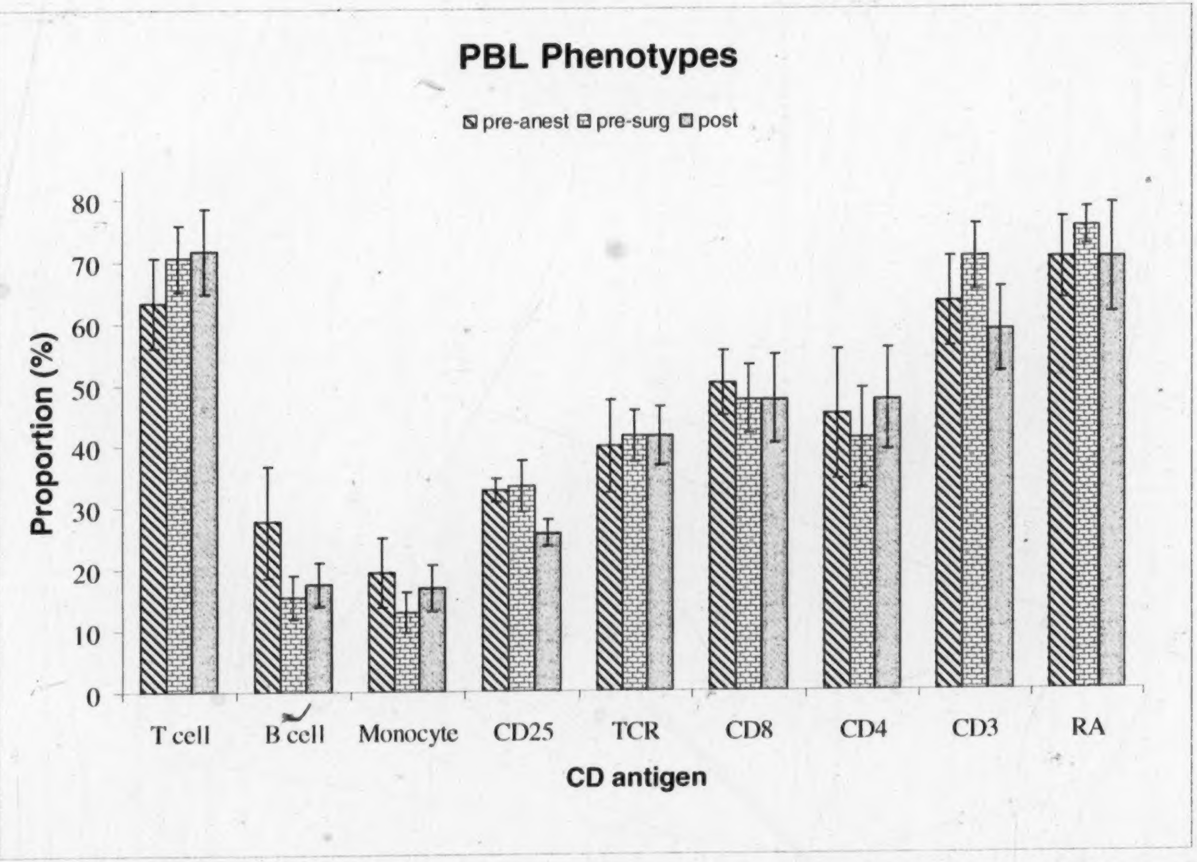


Figure 5. Effect of the surgical procedure on blood phenotypes. Immune phenotypes were identified in piglets (n=5) using specific anti-swine monoclonal antibodies by flow cytometry. Blood samples were taken at three time points: prior to administration of anesthetic (pre-anest), subsequent to administration of anesthetic but prior to surgery (pre-surg) and post-surgery (post). Significant comparisons were performed by ANOVA and no significant differences were found.

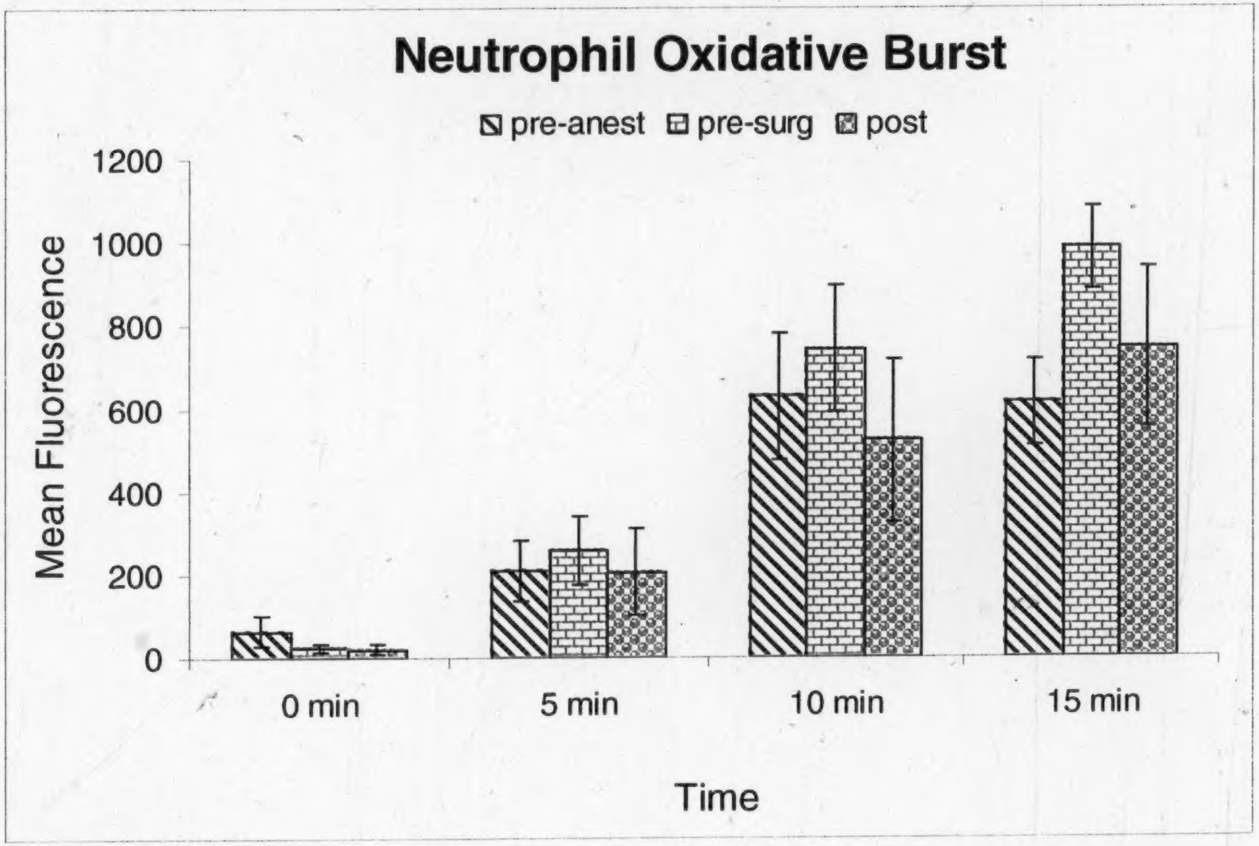


Figure 6. Blood neutrophil oxidative burst in animals (n=5) undergoing the *in situ* surgical model. Blood samples were taken at three time points: prior to administration of anesthetic (pre-anest), subsequent to administration of anesthetic but prior to surgery (pre-surg) and following the surgical procedure in which E. coli was inoculated into intestinal loops (post). Significant comparisons were performed by ANOVA and no significant differences were found between time points.

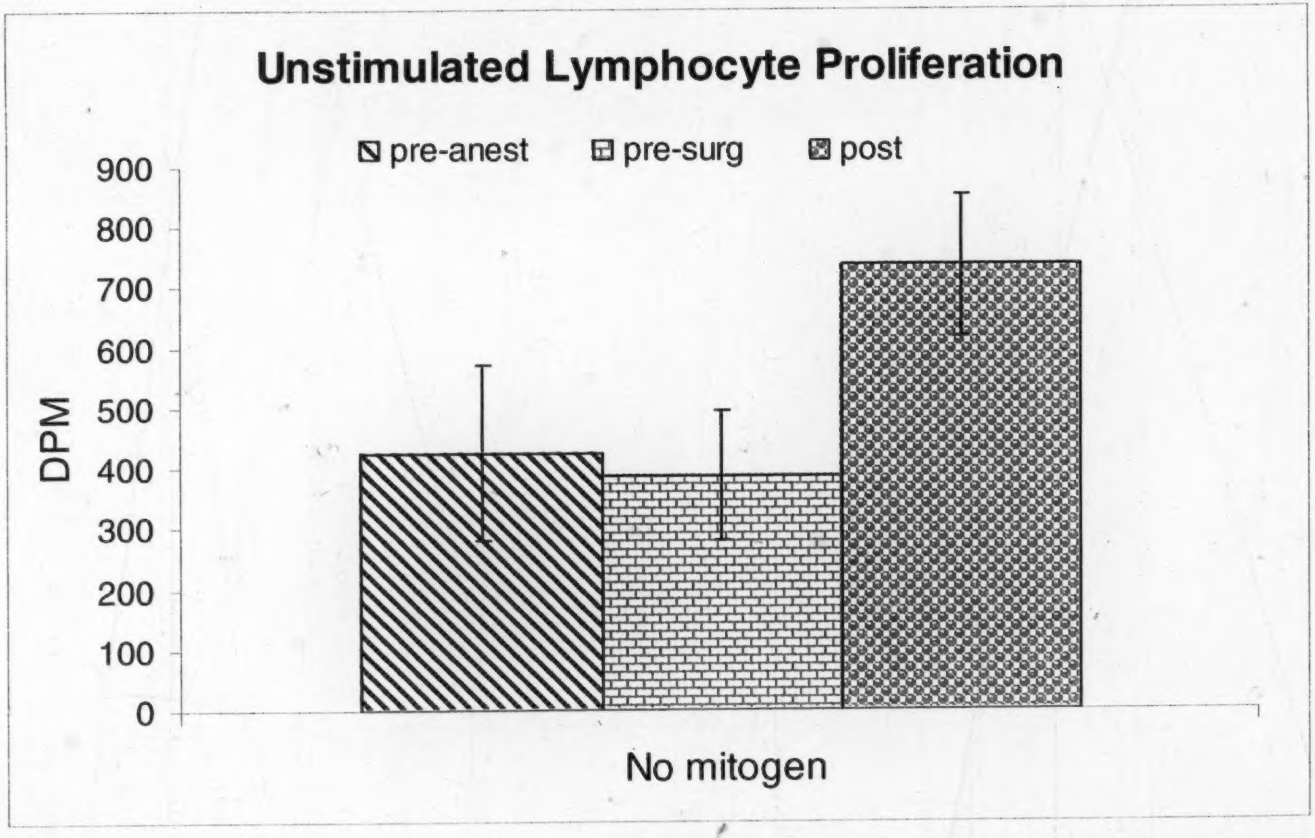


Figure 7. Proliferation of PBL lymphocytes 48 h following incubation in the absence of mitogen. Blood samples were taken at three time points: prior to administration of anesthetic (pre-anest), subsequent to administration of anesthetic but prior to surgery (pre-surg) and post-surgery (post). Significant comparisons were performed by ANOVA and no significant differences were found between time points.

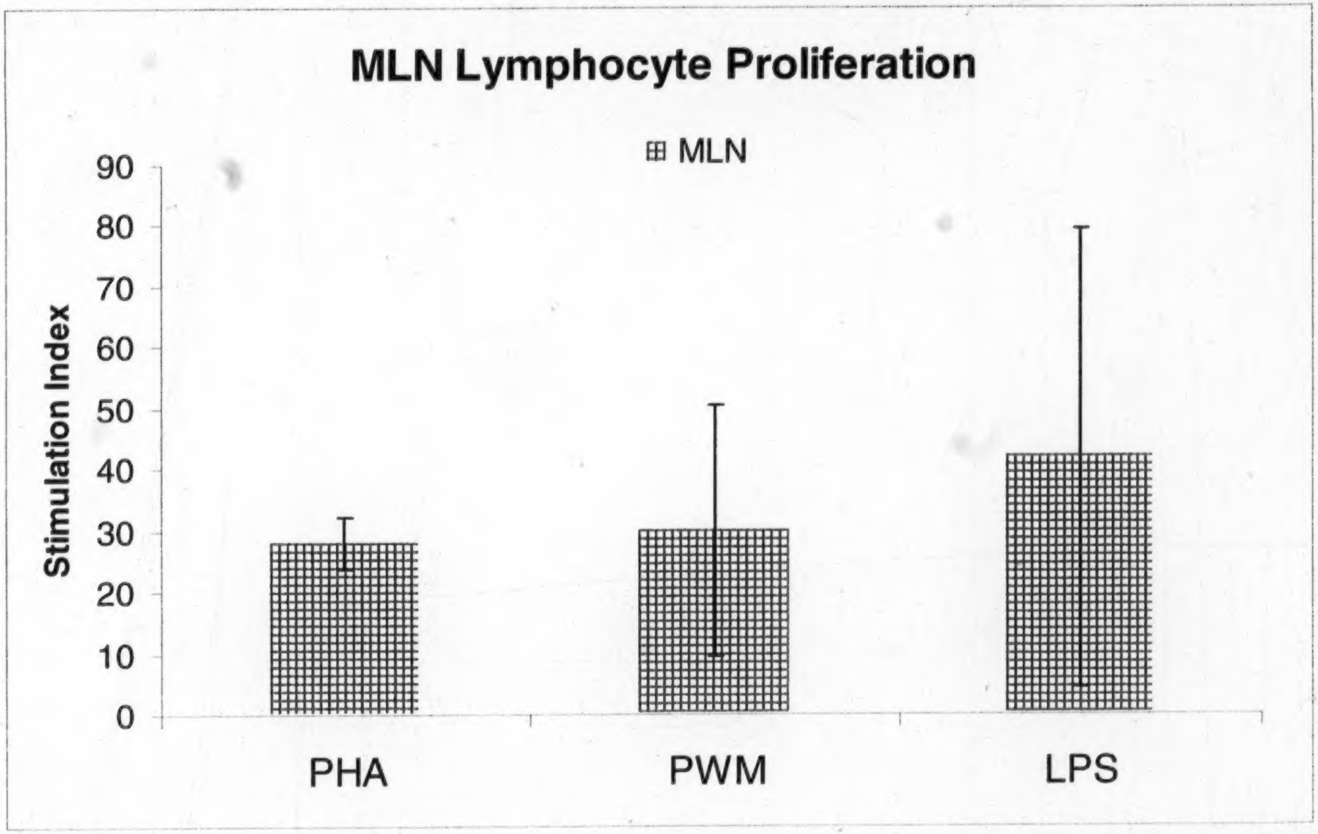


Figure 8. Proliferation of MLN lymphocytes 48 h following incubation with mitogen (PHA, PWM & LPS) and isolated post surgery (n=53). Significant comparisons were performed by ANOVA and significant differences are indicated with different letters. Despite the possible immunosuppressive effects of general anesthetic and surgery, cells responded in vitro to mitogen stimulation.

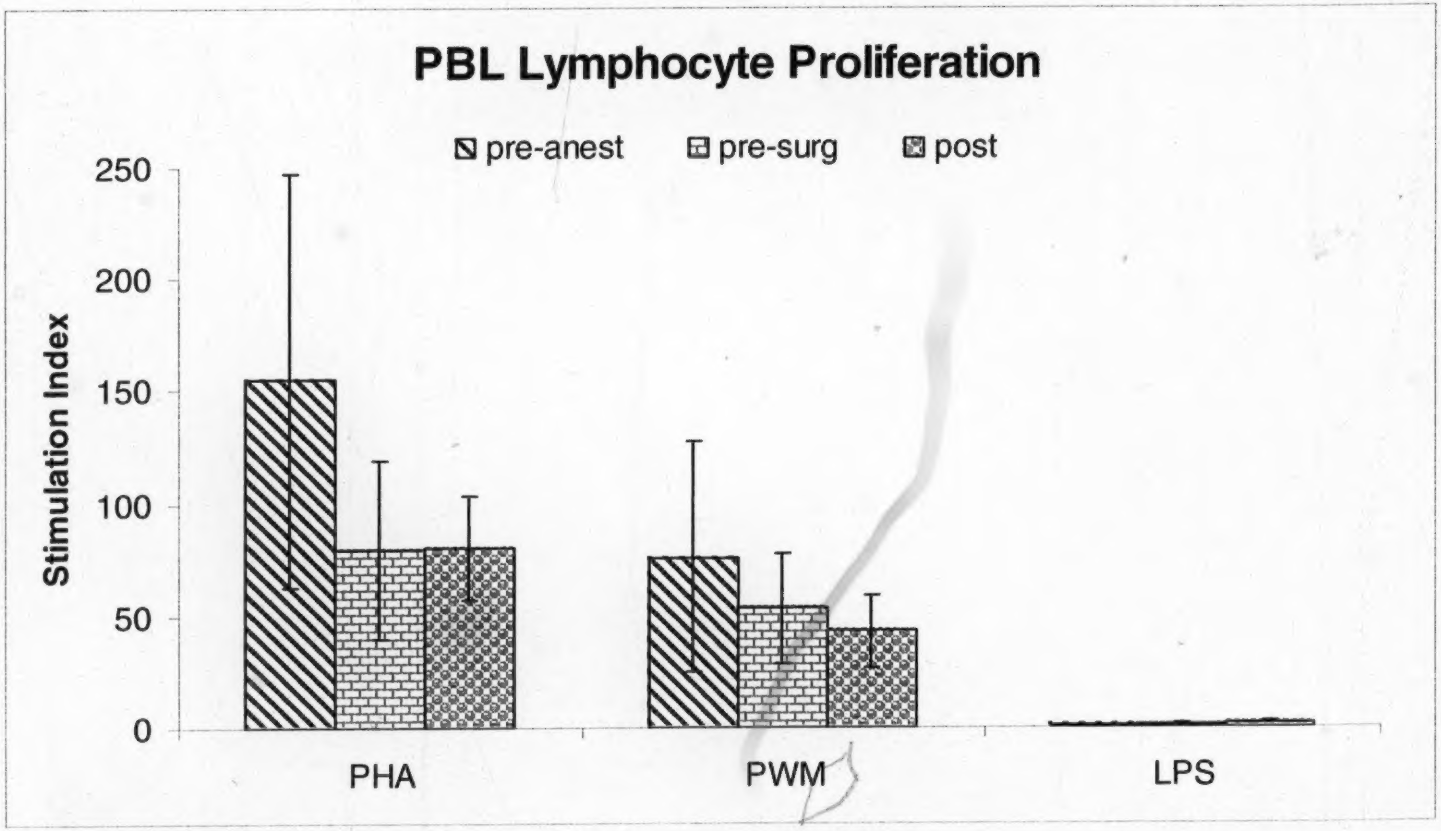


Figure 9. Proliferation of PBL 48 h following stimulation with mitogen and sampled at 3 time points: prior to administration of anesthetic (pre-anest), subsequent to administration of anesthetic but prior to surgery (pre-surg) and post-surgery (post). Significant comparisons were performed by ANOVA and no significant differences were identified between time points.

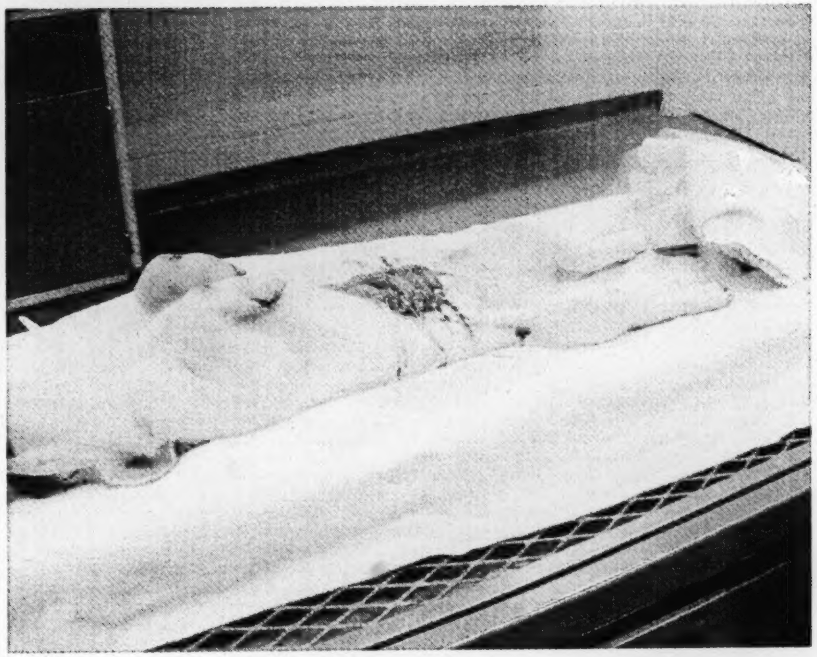
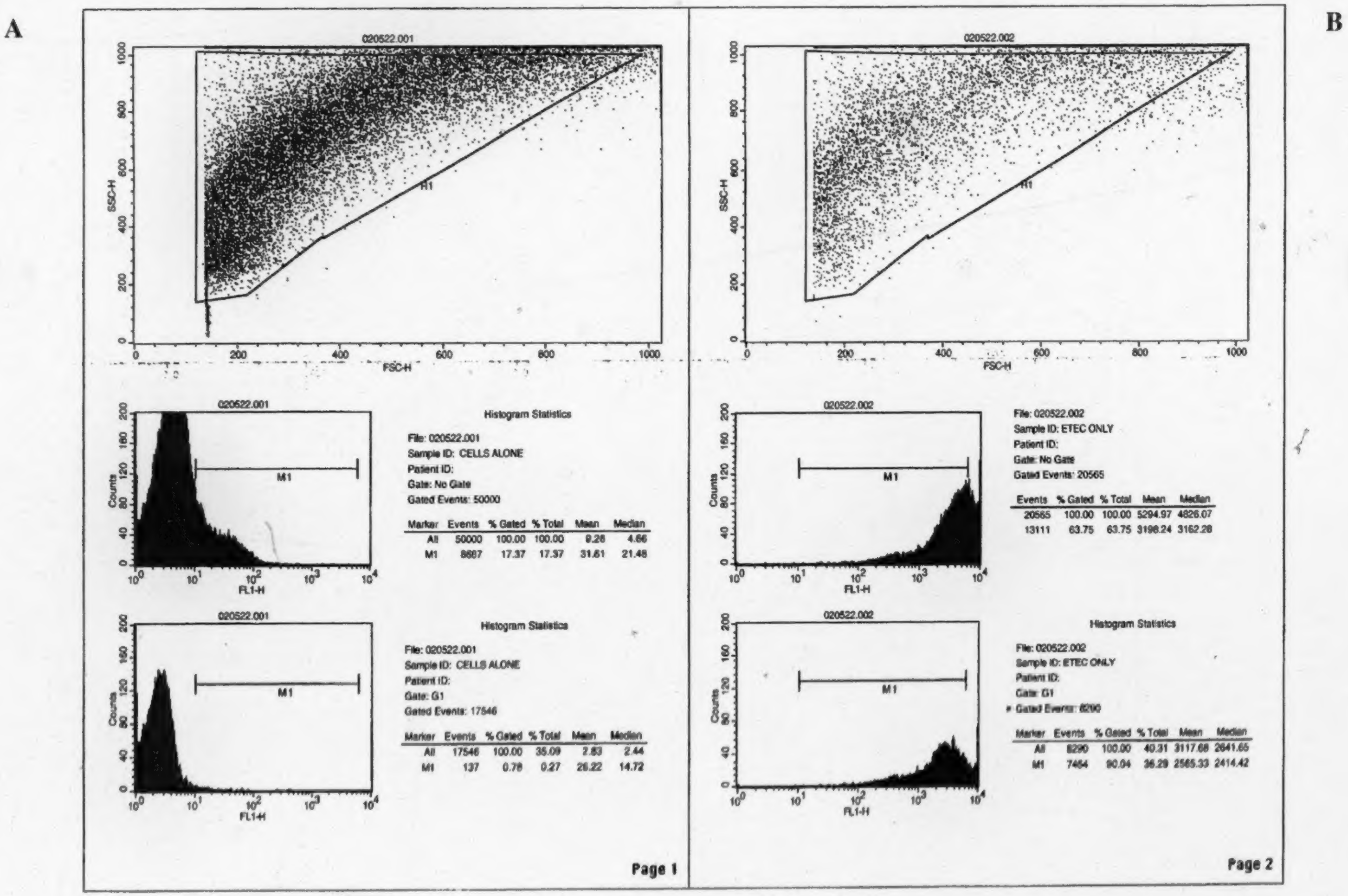


Figure 10. Photograph of in situ surgical procedure showing isolated, tied off intestinal loops.



```
Histogram Statistics

File: 020522.001
Sample ID:  CELLS ALONE
Patient ID:
Gate: No Gate
Gated Events: 50000

Marker  Events  % Gated  % Total    Mean   Median
   All   50000   100.00   100.00    9.26     4.66
    M1    8687    17.37    17.37   31.61    21.48
```

```
Histogram Statistics

File: 020522.001
Sample ID:  CELLS ALONE
Patient ID:
Gate: G1
Gated Events: 17546

Marker  Events  % Gated  % Total    Mean   Median
   All   17546   100.00    35.09    2.83     2.44
    M1     137     0.78     0.27   26.22    14.72
```

```
File: 020522.002
Sample ID: ETEC ONLY
Patient ID:
Gate: No Gate
Gated Events: 20565

Events  % Gated  % Total     Mean   Median
 20565   100.00   100.00  5294.97  4826.07
 13111    63.75    63.75  3198.24  3162.28
```

```
Histogram Statistics

File: 020522.002
Sample ID: ETEC ONLY
Patient ID:
Gate: G1
# Gated Events: 8290

Marker  Events  % Gated  % Total     Mean   Median
   All    8290   100.00    40.31  3117.68  2641.65
    M1    7464    90.04    36.29  2565.33  2414.42
```

Figure 11. Flow cytometry analysis of mucosal cell populations (A – Cell Alone) and of K88 ETEC populations (B – ETEC Only).

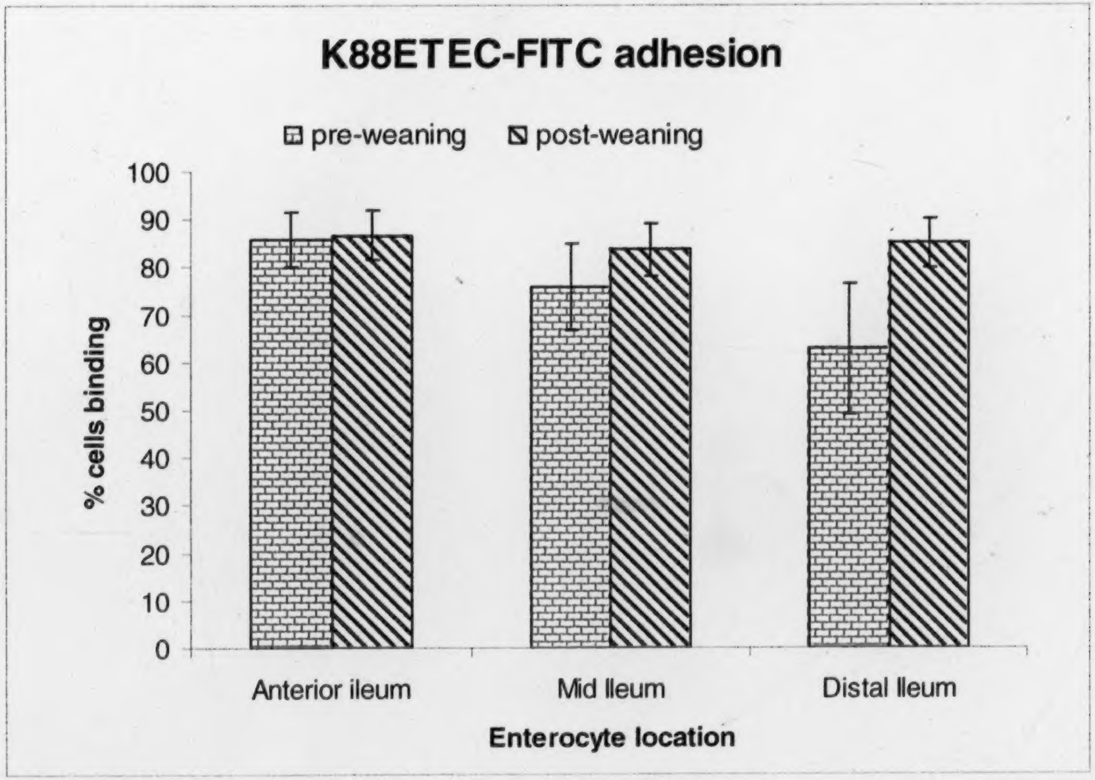


Figure 12. Percent of cells binding in ETEC adhesion assay of mucosal cells obtained from the piglet (n=10) anterior-, mid- and distal-ileum.

3.6 REFERENCES

Adegoke,O.A., McBurney,M.I., and Baracos,V.E. 1999a. Jejunal mucosal protein synthesis: validation of luminal flooding dose method and effect of luminal osmolarity. Am. J. Physiol 276: G14-G20.

Adegoke,O.A., McBurney,M.I., Samuels,S.E., and Baracos,V.E. 1999b. Luminal amino acids acutely decrease intestinal mucosal protein synthesis and protease mRNA in piglets. J. Nutr. 129: 1871-1878.

Adegoke,O.A., McBurney,M.I., Samuels,S.E., and Baracos,V.E. 2003. Modulation of intestinal protein synthesis and protease mRNA by luminal and systemic nutrients. Am. J. Physiol Gastrointest. Liver Physiol 284: G1017-G1026.

Andersen,J.K., Takamatsu,H., Oura,C.A., Brookes,S.M., Pullen,L., and Parkhouse,R.E. 1999. Systematic characterization of porcine ileal Peyer's patch, I. apoptosis-sensitive immature B cells are the predominant cell type. Immunology 98: 612-621.

Anup,R. and Balasubramanian,K.A. 2000. Surgical stress and the gastrointestinal tract. J. Surg. Res. 92: 291-300.

Bozkurt,M.K., Agalar,F., and Akcakanat,A. 2000. Inflammatory response to open tension-free inguinal hernioplasty versus conventional repair. Am. J. Surg. 179: 76.

Castejon-Casado,J., Moreno-Prieto,M., Balladares-Mendias,J.C., Alaminos-Mingorance,M. Lopez-Candel,E., and Ramirez-Navarro,A. 2001. Hormonal responde to surgical stress in schoolchildren. European Journal of Pediatric Surgery 11: 44-47.

Clarke,J.M. and Morton,R.J. 2000. Development of an in vitro fluorometric assay to study adherence of Pasteurella haemolytica to bovine cells. Am. J. Vet. Res. 61: 129-132.

Conway,P.L., Welin,A., and Cohen,P.S. 1990. Presence of K88-specific receptors in porcine ileal mucus is age dependent. Infect. Immun. 58: 3178-3182.

Dean,E.A., Whipp,S.C., and Moon,H.W. 1989. Age-specific colonization of porcine intestinal epithelium by 987P-piliated enterotoxigenic Escherichia coli. Infect. Immun. 57: 82-87.

Dugan,M.E. and McBurney,M.I. 1995. Luminal glutamine perfusion alters endotoxin-related changes in ileal permeability of the piglet. JPEN J. Parenter. Enteral Nutr. 19: 83-87.

Fan,M.Z., Stoll,B., Jiang,R., and Burrin,D.G. 2001. Enterocyte digestive enzyme activity along the crypt-villus and longitudinal axes in the neonatal pig small intestine. J. Anim Sci. 79: 371-381.

Field,C.J. 1996. Using immunological techniques to determine the effect of nutrition on T-cell function. Can. J Physiol Pharmacol. 74: 769-777.

Francis,D.H., Grange,P.A., Zeman,D.H., Baker,D.R., Sun,R., and Erickson,A.K. 1998. Expression of mucin-type glycoprotein K88 receptors strongly correlates with piglet susceptibility to K88(+) enterotoxigenic Escherichia coli, but adhesion of this bacterium to brush borders does not. Infect. Immun. 66: 4050-4055.

Girardeau,J.P. 1980. A new in vitro technique for attachment to intestinal villi using enteropathogenic Escherichia coli. Ann. Microbiol. (Paris) 131B: 31-37.

Guieu,R., Dufour,H., Devaux,C., Brue,T., Rosso,J.P., Grisoli,F., Grino,M., Enjalbert,A., Begoud,D., Broder,N., Rochat,H., and Jaquet,P. 1998. The hormonal response to stress is not modified by the dramatic decrease in prolactin plasma concentration during surgery for microprolactinoma. J. Neurol. Neurosurg. Psychiatry 65: 502-507.

Hall,G.M. and Ali,W. 1998. The stress response and its modification by regional anaesthesia. Anaesthesia 53 Suppl 2: 10-12.

Helmy,S.A., Wahby,M.A., and El Nawaway,M. 1999. The effect of anaesthesia and surgery on plasma cytokine production. Anaesthesia 54: 733-738.

Jepson,M.A. and Clark,M.A. 1998. Studying M cells and their role in infection. Trends Microbiol. 6: 359-365.

Kotani,N., Hashimoto,H., Sessler,D.I., Yasuda,T., Ebina,T., Muraoka,M., and Matsuki,A. 1999. Expression of genes for proinflammatory cytokines in alveolar macrophages during propofol and isoflurane anesthesia. Anesth. Analg. 89: 1250-1256.

Lahteenmaki,M., Salo,M., and Tenovuo,J. 1998. Mucosal host defence response to hysterectomy assessed by saliva analyses: a comparison of propofol and isoflurane anaesthesia. Anaesthesia 53: 1067-1073.

Larsen,J.W.A., Anderson,N., and Vizard,A.L. 1999. The pathogenesis and control of diarrhoea and breech soiling in adult Merino sheep. Int. J. Parasit. 29: 893-902.

Laux,D.C., McSweegan,E.F., and Cohen,P.S. 1984. Adhesion of enterotoxigenic *Escherichia coli* to immobilized intestinal mucosal preparations: a model for adhesion to the mucosal surface components. J. Microbiol. Meth. 2: 27-39.

Madsen,K.L., Yanchar,N.L., Sigalet,D.L., Reigel,T., and Fedorak,R.N. 1995. FK506 increases permeability in rat intestine by inhibiting mitochondrial function. Gastroenterology 109: 107-114.

Markovic,S.N., Knight,P.R., and Murasko,D.M. 1993. Inhibition of interferon stimulation of natural killer cell activity in mice anesthetized with halothane or isoflurane. Anesthesiology 78: 700-706.

Markovic,S.N. and Murasko,D.M. 1993. Anesthesia inhibits interferon-induced natural killer cell cytotoxicity via induction of CD8+ suppressor cells. Cell Immunol. 151: 474-480.

Mobert,J., Zahler,S., Becker,B.F., and Conzen,P.F. 1999. Inhibition of neutrophil activation by volatile anesthetics decreases adhesion to cultured human endothelial cells. Anesthesiology 90: 1372-1381.

Nabuurs,M.J. 1998. Weaning piglets as a model for studying pathophysiology of diarrhea. Vet. Q. 20 Suppl 3: S42-S45.

Pabst,R., Geist,M., Rothkotter,H.J., and Fritz,F.J. 1988. Postnatal development and lymphocyte production of jejunal and ileal Peyer's patches in normal and gnotobiotic pigs. Immunology 64: 539-544.

Roumen,R.M.H., Van Der Vliet,J.A., Wevers,R.A., and Goris,R.J.A. 1993. Intestinal permeability is increased after major vascular surgery. J. Vasc. Surg. 17: 734-737.

Sarmiento,J.I., Casey,T.A., and Moon,H.W. 1988. Postweaning diarrhea in swine: experimental model of enterotoxigenic Escherichia coli infection. Am. J. Vet. Res. 49: 1154-1159.

Sellwood,R. 1979. Escherichia coli diarrhoea in pigs with or without the K88 receptor. Vet. Rec. 105: 228-230.

Snodgrass,D.R., Chandler,D.S., and Makin,T.J. 1981. Inheritance of Escherichia coli K88 adhesion in pigs: identification of nonadhesive phenotypes in a commercial herd. Vet. Rec. 109:461-463.

Stokes,C.R., Haverson,K., and Bailey,M. 1996. Antigen presenting cells in the porcine gut. Vet. Immunol. Immunopathol. 54: 171-177.

Thorns,C.J., Sojka,M.G., and Roeder,P.L. 1989. Detection of fimbrial adhesins of ETEC using monoclonal antibody-based latex reagents. Vet. Rec. 125: 91-92.

Vowells,S.J., Sekhsaria,S., Malech,H.L., Shalit,M., and Fleisher,T.A. 1995. Flow cytometric analysis of the granulocyte respiratory burst: a comparison study of fluorescent probes. J. Immunol. Methods 178: 89-97.

Chapter 4 – IMMUNE DEVELOPMENT OF PIGLETS FROM EARLY WEANING AT THREE WEEKS OF AGE TO FIVE WEEKS OF AGE

4.1 INTRODUCTION

Newborn piglets face a great variety of challenges in their first five weeks, including the increasing practice of early weaning from maternal milk to grain-based diets. Piglets were formerly weaned at ten to twelve weeks old, when they were accustomed to creep feeding because the amount of milk a sow produced decreased with time and was almost negligible by ten to twelve weeks of age (Nabuurs 1998). A more modern practice is weaning at an age of three to five weeks, when piglets are abruptly switched from sow's milk to a weaning diet and this stress can result in adverse reactions, including the onset of diarrhea (Nabuurs 1998). The critical importance of the first few weeks of a piglets' life is demonstrated in the fact that the majority of losses to the swine industry occur at this young age (Patience 1989). It has been stated that the continuous presence of orally obtained antibodies through sow's milk is of importance for immune protection as the piglets immune system matures (Valpotic et al. 1989).

Although lymphoid cell subpopulations have been characterized for the mature pig (Jonjic et al. 1987; Lunney & Pescovitz 1987; Saalmuller et al. 1989), less is known about the neonatal period (Becker & Misfeldt 1993). Both the weaning process and its timing have been reported to significantly influence the expression of lymphocyte surface antigens (Bailey et al. 1994; Bailey et al. 1998; Bianchi et al. 1992; Hampson 1986; Solano-Aguilar et al. 2001) and appears to be more pronounced near the gut (Solano-Aguilar et al. 2001). The swine immune system is reported to have some distinct differences from other species. For example, they have a more diversified T lymphocyte population than humans (Saalmuller et al. 1989). The T lymphocytes (T cells) population in swine consists of CD2+ (60% of PBL 18-19 d of age and 35% at 27-30 d of age) and CD2- subsets (Becker & Misfeldt 1993), as well as a double positive CD4+CD8+ T cell subset (Rodriguez-Carreno et al. 2002; Saalmuller 1989). The percentage of these double positive T cells in blood is reported to start very low (<2% in at 3 d) and gradually increases over the first year of life (up to 16% of total lymphocytes (Pescovitz et al. 1994))(Summerfield et al. 1996; Zuckermann & Gaskins 1996). Based on the expression of cell surface antigens and the size of the cells, CD4+CD8+ T cells are thought to belong to a mature resting population of lymphocytes (Pescovitz et al. 1990; Saalmuller et al. 1987a; Saalmuller et al. 1987b; Saalmuller et al. 1989; Saalmuller & Bryant 1994; Summerfield et al. 1996). Dual expression of these two co-receptors is hypothesized to affect function and antigen recognition (Pescovitz et al. 1994).

GALT is the largest immune organ of the body and responsible for handling large quantities of potentially harmful antigens (Insoft et al. 1996) along the length of the intestinal tract. By the time piglets are born, a detectable number of immature B lymphocytes (B cells) are present in the lymph nodes and Peyer's patches (PP: Bianchi et al. 1992; Pabst & Rothkotter 1999). However, between days 1 and 42, there is a large increase (15-fold) in the cell production rate in the piglets ileal PP (Stokes et al. 1996) and the PP has been shown to increase in size several times following birth (Chu et al. 1979).

The objective of this study was to determine changes in immune development in the peripheral and gut immune tissues (between 3 and 5 weeks of age). Development in innate immune defense was defined by changes in neutrophil function and improvement in adaptive defense was defined as an increased response to mitogens in PBL, MLN lymphocytes and PP lymphocytes, an increased production of specific cytokines from these mitogen-stimulated lymphocytes and changes in immune cell populations that reflect maturation.

4.2 MATERIALS AND METHODS

Animals and Diets

Animal protocols were reviewed and approved by the Faculty of Agriculture and Forestry Animal Policy and Welfare Committee and were conducted in accordance with the Canadian Council on Animal Care guidelines. Ten female and ten male Dutch-Landrace (Genex swine group, Heartland Livestock Services, Regina, Saskatchewan) piglets (5538 g ± 325) from 5 separate litters were obtained and weaned at approximately 21 days of age from the University of Alberta Swine Research and Technology Centre. Piglets were transported to a separate, temperature controlled room at the University of Alberta Metabolic Research Unit where they were housed individually in metabolic crates, each fitted with water nipples and creep feeders. Upon receipt of each litter, piglets were assorted randomly into baseline or weaned to a basal diet that was fed for approximately two weeks. The feeding design is shown in the Appendix (Figure 2) and diets were formulated to meet 110% of the requirements for piglets 5-10 kg as specified by the National Research Council (NRC 1998). A constant portion of the diet is detailed (Table

1) and contained 65 g/kg of fat from a mixture of sources (59.7% Tallow, 33.1% Safflower Oil, and 7.2% Linseed Oil), providing a polyunsaturated to saturated fatty acid ratio of ~ 0.9. The supplement portion of the diet (60 g/kg) consisted of consisted of a mixture of amino acids (21.0 g/kg serine, 17.8 g/kg alanine, and 15.0g/kg glycine), which are not known to be limiting for growth or immune function in piglets of this age. Animals assigned to the 21 d and 35 d groups were given access to water and a small amount of basal diet overnight. Prior to sample collection, anesthesia was induced in these piglets (pre-weaning, n=10), along with animals at approximately 35 days of age (post-weaning, n=10) by intramuscular injections of Torbugesic (0.2 mg/kg), Ketamine (11 mg/kg), Rompun (2.2 mg/kg), and Robinul (0.01 mg/kg) and maintained with 1.0-1.5% v/v halothane delivered with 3L/min oxygen. Samples were collected in a separate, closed surgery room following Euthanasia by cardiac injection of Pentobarbital Euthanyl (2 ml/4.5 kg body weight (BW)).

Isolation of Immune Cells

Blood samples and MLN were sampled as described previously (Chapter 3). PBL and MLN lymphocytes were isolated from these samples as previously described (Chapter 3). Immune cells were also isolated from PP according to methods described by Solano Aguilar et al. (2000) (Solano-Aguilar et al. 2001). Briefly, excess mesenteric and fat tissues were removed from the intestine. The intestine was rinsed 2 x with Hank's Balanced Salt Solution (HBSS) + dithiothreitol (DTT; Sigma; Oakville, ON) buffer (filtered 0.22 um and pH 7.2, HBSS without Ca and Mg; DTT 2 mM final concentration; Hepes 0.01 final concentration). The intestine was cut down the mesenteric line and the

PP was dissected out. The entire patch was dissected into small pieces and incubated 20-30 min. at 37°C in 20 mls HBSS + DTT buffer. The supernatant was discarded and the cells were incubated an additional 20-30 min. in HBSS + Ethylenediaminetetraacetic acid (EDTA; Sigma; filtered 0.22 *u*m and pH 7.2. HBSS without Ca and Mg; EDTA 1 mM final; Hepes 1 mM final). The supernatant was discarded and cells were passed through a sterile 100 *u*m mesh nylon screen using the barrel of a sterile syringe and buffer (1% (v/w) Bovine Serum Albumin (BSA; Sigma) in Phosphate Buffered Saline (PBS)). The sample was then pelleted at 450 x g for 5 min, supernatant was discarded and the pellet re-suspended in 6 mls 40% v/v Percoll (Pharmacia Biotech, Baie D'Urfe, Quebec) which was layered over 6 mls 70% v/v Percoll. The gradient was then spun 750 x g for 30 min. Immune cells were removed from the interface, pelleted at 450 x g for 5 mins and re-suspended in Complete Culture Media (CCM) to be used for counting and performing assays.

Immune Measures

The mitogen response assay was conducted in triplicate on microtiter plates as previously described (Chapter 3). Additionally, immune cells from blood and MLN in CCM (1 x 10^6 cells/ml) were either stimulated with PHA (25 *u*g/ml) or incubated in the absence of mitogen in sterile plastic tubes (Fisher) for 48 h in a humidified atmosphere at 37^0C in the presence of 5% CO_2. This incubation time was chosen based on previous studies conducted in piglets in which the peak production of IL-2 was found by cells stimulated with PHA. Supernatants were collected and frozen at -70^0C for later determination (ELISA, Medicorp, Montreal, Quebec) of production of the following cytokines: Th1 type cytokines IL-2, IFN-γ, TNF-α) and Th2 type cytokines (IL-10 and IL-4). These assays were performed according to the manufacturer instructions. Absorbance was read at 450 nm by spectrophotometry (SpectraMax190, Molecular Devices, Sunnyvale, CA). Standard curves were generated with the following standard concentrations: 8.8-570 pg/mL (IL-2), 15.8-1000 pg/mL (IL-4), 62.5-2000 pg/mL (IL-10), 15.6-1000 pg/mL (TNF-α) and 31.3-1000 pg/mL (IFN-γ). Confidence value (CV) intervals were less than 15% for all cytokines tested. Lymphocyte subsets from freshly isolated immune cells in blood, MLN and PP were identified by indirect immunofluorescence assay as previously described (Chapter 3; Ab description given in Chapter 1; antibody profile given in Table 3 of Appendix). Lymphocytes isolated from MLN and PP were added to each well to a concentration of 2-5 x 10^5 cells/well. Whole blood was used to assess neutrophil function, as described (Chapter 3).

Statistics

All statistical analyses were completed using the SAS statistical package (Version 8.1, SAS Institute, Cary, NC). The age comparison was performed by one-way analysis of variance, blocked for litter. All results were presented as means ± SEM. Kurtosis and Skew were analyzed for both proliferation and cytokine data. Values that were not considered normally distributed as determined by skew and kurtosis were log transformed prior to statistical analysis and are indicated in respective tables as log transformed.

4.3 RESULTS

Intake and Weight Gain

On average, piglets supplemented for two weeks gained 1515 ± 289 g and had an average final BW of 7093 ± 674 g. Feed intake (FI) averaged 396 ± 156 g/d and 5837 ± 435 g/kg BW.

Neutrophil Function

No significant differences in neutrophil oxidative burst, size or granularity were found as the piglets aged (Table 2).

Changes in Cell Phenotypes with Age

The percentage of CD3+ T cells was lower (18% decrease) at 35 d of age in blood but was not caused by changes in the proportion of CD3+CD4+ cells or CD3+CD8+ cells, as these populations were not significantly changed as the piglets aged (Table 3). An age-related decrease in the proportion of CD2+ cells was also found in PBL (Figure 1) due to a decrease in CD2+CD3+ cells (49% decrease). T cell expression of adhesion molecules was significantly increased with piglet age (32% increase in CD3+CD44+ cells) (Table 3). There were no changes in the proportion CD45RA+ or CD45RA- T cells in blood (Figure 2). While there were no significant changes in the percentage of B cells, immunoglobulin (Ig) A+ cells or IgM+ cells, there was a small but significant increase in the percentage of B cells expressing CD44 (1% increase) with age (Table 3). There were also no changes in the proportion of blood monocytes (Table 3).

There was no change in the percentage of CD3+ cells, CD2+ cells, or any subsets of activation markers in MLN (Table 4). An age-related increase in the proportion of total B cells (28% increase) and IgA+ cells (41% increase) were found in MLN, with no change in the proportion of IgM+ cells (Figure 3). There was a significant increase in total RA+ cells (9% increase) in MLN that was likely influenced by the significant age-related increase in the percentage of CD4+ cells expressing the CD45RA antigen (Figure 4).

In PP, the observed increase in CD3+ cells (44% increase) could be attributed to the slight, but non-significant increases in the proportion of CD2+, CD4+, and CD8+ cells (Figure5). There was a small increase in the proportion of CD4+CD45RA- cells (20% increase) with piglet age. The significant decrease in total CD45RA expression (8% decrease) was not likely caused by T cells as the proportion of CD4+CD45RA+ and CD8+CD45RA+ cells did not significantly change with age (Figure 6). The only significant change in the B cell population in this tissue was an increase in PP Bcell+ cells (55% increase) with no changes in the proportion of monocytes (Table 5).

Effect of Age on Lymphocyte Proliferation

With age, PBL (but not MLN and PP cells) incubated in the absence of mitogen incorporated less ^{3}H-thymidine ($p<0.01$) (Table 6). There was an increase in both the absolute rate of ^{3}H-thymidine uptake to PHA (Figure 7) and PWM (Figure 8) and the stimulation index (Table 6) with age (PHA <0.02, PWM $p<0.005$). A significant age-related decrease in DPM following stimulation of PBL with lipopolysaccharide (LPS) ($p<0.0001$) was found (Figure 9), but this difference was not significant when corrected for the un-stimulated response at 35 days (Table 6). In comparison to 21 day-old piglets, incorporation of ^{3}H-thymidine (DPM) in MLN from 35 day-old piglets was significantly increased in response to PHA ($p<0.0001$)(Figure 7), PWM ($p<0.005$) (Figure 8) and LPS ($p<0.05$)(Figure 9). MLN also had a 1.7-fold greater SI in response to PWM ($p<0.05$)(Table 6), and although there was also an increase in the SI response to PHA and LPS, the increase was not significant. The response of PP cells to mitogens was quite low compared to PBL and MLN and there was high intra-individual variation and no differences were found between groups (Table 6).

Cytokine Production

Despite the higher proliferative response to PHA by cells from MLN and blood few changes were observed in the production of cytokines following mitogen stimulation (Tables 7 and 8). In PBL, the only significant difference observed was a decrease in the IFN-γ/IL-4 ratio with age (Table 7). In MLN, consistent with the higher ^{3}H-thymidine response there was a higher production of IL-2 with age (Table 8).

4.4 DISCUSSION

Wheat (38%) and soybean meal (23%) were the major dietary ingredients used in this study, which is typical for western swine production (Patience & Thacker 1989). Hypersensitivity was a concern as previously feeding soybean meal to piglets decreased villus height (Li et al 1990; Dréau et al. 1994), increased serum IgG (Li et al 1990), decreased growth performance (Friesen et al. 1993), and increased susceptibility to infection (Dréau et al. 1994). Studies in piglets weaned at 3 weeks suggest that inappropriate immune responses can be mounted to soy antigens, but these responses are controlled by oral tolerance mechanisms (reviewed in (Bailey et al. 2002)). In fact, soybean meal has been shown to be well tolerated by swine and weaning diets containing up to 34 percent soybean meal have been used without affecting growth performance (Lawrence et al. 2003). As a precaution, the amount of soy fed to piglets in the current study was limited by including casein as an additional protein source. Although the amount of feed consumed by piglets was initially low, feed intake increased on a daily basis up until the conclusion of the trial. Comparison of innate immune function in suckled- and diet-fed piglets (2 weeks post-weaning) revealed that age did not alter neutrophil function both un-stimulated (time 0) and following mitogen stimulation. This is consistent with a comparison of neutrophils isolated from newborn piglets compared to piglets at 6 weeks of age (Hoskinson et al. 1990) and confirms that this aspect of the innate immune system is well developed at weaning.

Peripheral Lymphocyte Distribution and Function

To test the hypothesis that adaptive immune function increased 2-weeks post-weaning, lymphocyte proliferation and cytokine production were measured and immune cell populations were characterized. With age *in vivo* (unstimulated) incorporation of ^{3}H-thymidine decreased. The higher un-stimulated mitogen response in young suckled piglets in the current study is consistent with previous reports when newborn piglets were compared to those at 6 wks of age (Hoskinson et al. 1990). Furthermore, this *in vivo* activity of lymphocytes has also been observed in human infants (Field et al. 2001) and in the young piglet may be related to immune compounds provided in sow's milk (Vega-

Lopez et al. 1995). An increased ability of PBL from older animals to respond to mitogen has been demonstrated previously, which could be detected from day 1 to 16, but were greater when compared to piglets at 28 days of age (Becker & Misfeldt 1993). These findings were also supported by other studies in pigs, where the responsiveness of PBLs to T cell mitogens has been reported to increase during the first few weeks after birth (Becker & Misfeldt 1993; Blecha et al. 1983; Hoskinson et al. 1990; Valpotic et al. 1989). In this study, when PBL were incubated in the presence of mitogen, there was a significant age-related increase in incorporation of ^{3}H-thymidine in response to PHA and PWM (10-fold greater SI). This finding is interesting because there were significant decreases in the proportion of total T cells (CD2+) and CD3+ (TCR complex) cells with age, which was reflected in a significant decrease in the proportion of CD2+CD3+ cells. The decrease in total T cells would be expected along with decreases in CD3+ cells, since mature peripheral T cells express this antigen as a component of the TCR complex. Since several components of the CD3 complex are essential in pre-TCR assembly and function (Haks et al. 1999) during T cells development in the thymus, it's expression will vary according to the total proportion of T cells in PBL.

Interestingly, PBL from older pigs had significantly decreased (71%) rate of ^{3}H-thymidine uptake in response to LPS, despite no apparent change in the proportion of B cells. This mitogen is recognized by specific B cell receptors and stimulation of these receptors induces B cell resistance against apoptosis, upregulation of costimulatory molecules, and proliferation (Miyake 2003). Since the proportion of B cells in this immune compartment is relatively small (22-24%; Table 3), the fact that PBL did not respond as well to a B cells mitogen such as LPS is not surprising. However, the reason why the response is decreased with age is unknown and may involve the higher in vivo activity of lymphocytes in younger piglets, which might have primed the cells.

A common immunological practice is the log transformation of cytokine data to account for non-normal distributions. The significant age-related decrease in the IFN-γ/IL-4 ratio in the current study suggests a shift in the Th1/Th2 balance towards Th2. Therefore, these findings suggest an increased ability of PBL to proliferate to T cell mitogens, even with a decreased proportion of T cells in an environment that would favor a humoral-mediated response. There was also an overall increased response in cytokine production to PHA. The apparent shift in the Th1/Th2 balance was supported by a 34% increase in IL-4 and 38% increase in IL-10 production (Th2), with a 52% decrease in IL-2- and 55% decrease in production of IFN-γ (Th1) (all non-significant). This shift in towards more of a Th2 response may have been influenced by changes in immune cell proportions in PBL. Previously, about one-third of CD4+ T cells produced IFN-γ at high levels, almost 50% synthesized IFN-γ within the CD8+ T cells population and there was no production association with either B cells or monocytes (Rodriguez-Carreno et al. 2002). Therefore, although it is not clear why these cells make more Th2 cytokines, the overall decrease in total T cells in PBL from this study may have contributed to the decreased IFN-γ/IL-4 ratio.

The adhesion molecule CD44 mediates lymphocyte migration by cell-cell adhesion, cell adhesion with extracellular matrix, endothelium, and other leukocytes (Arriens et al.

1998; Yang & Binns 1993). Anti-CD44 abs stain most human and porcine PBL (Yang & Binns 1993), which was also the case in this study, as 95% (Table 3) of PBL expressed this adhesion molecule compared to a much smaller number of PP lymphocytes (29-31% Table 5). In examining the specific cell populations that expressed CD44, there was a significant increase in PBL CD3+CD44+ and the percentage of B cells expressing CD44. Since upregulation of CD44 on B cells has been found to be characteristic of functionally effective B memory cells (Camp et al. 1991; Murakami et al. 1990) and expression of CD44 and CD2 molecules were both up-regulated on memory T cells (Sanders et al. 1988), these results suggest that that the proportion of memory B and T cells in the periphery of the piglet increases with age. This was somewhat predictable and potentially a result of increased antigen exposure as a result of the weaning process. To relate these findings to the increased proliferation of PBL to PHA and PWM, the greater proportion of CD3+CD44+ cells might have contributed to the higher response to PHA.

Mesenteric Lymph Nodes

Proliferation (measured by rate of ^{3}H-thymidine uptake) of MLN lymphocytes to all 3 mitogens was higher in piglets at 35 days of age. The increased PHA response was further supported by the increased production of IL-2 by MLN cells from 35 d-old piglets. IL-2 has an important role in supporting T lymphocyte proliferation (Choi & Yoo 2002). The age-related increase in MLN lymphocyte proliferative ability may have also been influenced by immune cell subset composition and the activation status of cell populations within the tissue microenvironment, which has been shown previously with CD25 expression (Whary et al. 1995). Although characterization of the major immune cell populations revealed no age-related differences in T cell population or activation status of these cells (CD25+), there were significant changes within the B cell compartment, with increases in the proportion of total B cell and IgA+ cells that were likely CD45RA+. This is because results from this study demonstrated a small, but significant increase (9.1%) in the proportion of CD45RA+ cells in MLN. Although expression of CD45RA on CD4+ and CD8+ T cells increased with age, the increase was not significant. Therefore, a separate population was contributing to this effect in MLN, possibly B cells, which also expresses CD45RA (Shivtiel et al. 2002). This is further supported by the fact that following activation, T cells undergo alternative splicing of CD45 to switch to expression of CD45RO (Braakman et al. 1991).

The percentage of B cells in MLN have been shown to remain stable as animals age (Solano-Aguilar et al. 2001) and both B and T cell populations in swine MLN were reported to be fully developed prior to birth with no observable difference between young and mature animals (Bianchi et al. 1992). Thus the findings of increased total B cells and IgA+ cells in the present study are contrary to these earlier findings. Interestingly, the proportion of IgM+ cells, from which IgA+ cells are generated, was not significantly influenced by age. One might predict that a higher proportion of IgA+ cells would result in a lower proportion of IgM+ cells. The finding of increased IgA+ cells in MLN suggests that a greater proportion of B cells are exposed to antigen at mucosal sites as piglets' age. An increased amount of antigen exposure was expected in these piglets, with the cessation of suckling and since they are introduced to new dietary antigens during the weaning process. Therefore, the results of this study suggest that the increase in IgA+

cells may be due to increased antigen processing within MLN, resulting from an increased maturation of B cells into IgA-secreting plasma cells (Insoft et al. 1996; Keren 1992). Previously, an increased proportion of Ig+ cells (Whary et al. 1995) and elevated antibody titres (Barnett et al. 1989) were found in MLN of piglets as a result of antigenic stimulation in this tissue by exposure to new dietary antigens.

Peyer's Patches

Characterization of the major immune cell populations in PP revealed that while there were no significant changes in the population of the classical CD4+CD8- T helper cells (Th) and CD8+CD4- cytotoxic/suppressor cells as the animals aged, the proportion of cells expressing the CD3 antigen was significantly increased. This increase was accompanied by a small, but non-significant increases in the proportion of CD2+, CD4+, and CD8+ cells and seems to indicate that the proportion of T cells in PP increases as piglets age. Further characterization of the PP T cell population revealed a decrease in the proportion of antigen naïve cells (CD45RA+). Thus, this study supports the hypothesis that piglet PPL encounter an increasing antigen load with age and weaning. In this study, the increasing proportion of antigen mature cells with age and weaning was supported by the finding of a significant increase in the proportion of CD4+RA- cells. However, when the percentage of CD4 and CD8 T cells expressing the RA+ isotype was calculated (Figure 6), there were small increases with age, but the changes were very small and did not reach significance. Therefore, a separate cell population, specifically the B cell population, which also expresses CD45RA (Shivtiel et al. 2002) might be responsible for the observed decrease in total expression.

Of major interest in PP is the predominant B cell population (46-53% of cells, Table 5). Previously, a reduction in the proportion of B cells (CD21) was shown in ileal PP with piglet age (Solano-Aguilar et al. 2001) and in the jejunal PP in piglets from 1.5 to 12 months of age (Rothkotter & Pabst 1989). The only significant change in the proportion of B cells was a PP-specific B cells, which was shown to increase. This antibody, which binds predominantly to ileal PP B cells (79-87%) identifies an immature population of B cells in the follicles of ileal PPs and is recognizable in lymph node as a B cell subpopulation with lower levels of surface Ig (Denham et al. 1998).

Incorporation of ^{3}H-thymidine by PP cells was low compared to PBL and MLN and there was high intra-individual variation. This variation was likely a result of environmental factors. This is supported by the lower variation in older piglets that were consuming the same diet. This low proliferative capacity of PP lymphocytes may have been influenced by immune cell populations in this tissue. Although the proportion of CD3+ cells significantly increased with piglet age, the proportion of CD3+ cells (14-25% of cells) in PP (Table 5), compared to blood (54-66% of cells; Table 3) and MLN (44-51% of cells; Table 4) was very low. Since the cytoplasmic domains of CD3 are involved in lymphocyte proliferation (Kirkham et al. 1996), a lower proportion of cells expressing the CD3 antigen in PP may have interfered with the ability of PP lymphocytes to proliferate following mitogenic stimulation, with the primarily T cell mitogens (PWM and PHA) used in the current study. An increased proportion of immature cells (PP Bcell) in PP may explain the observed (non-significant) decreases in PP cell ^{3}H-thymidine incorporation after stimulation with LPS (primarily a B cell mitogen).

Conclusion

It was hypothesized that piglet innate immune defense, demonstrated by changes in neutrophil function, at 35 days was more mature than at 21 days. The findings of this study suggest that this measure of innate immune function did not undergo many changes from pre-weaning to post-weaning, at least for peripheral neutrophil function. It was also hypothesized that piglet adaptive immune defense at 35 days is more developed than at 21 days. An improvement in adaptive immune defense was defined as a) an increased response to mitogens in lymphocytes isolated from blood, MLN and PP b) an increased production of specific cytokines from these mitogen-stimulated lymphocytes c) changes in immune cell populations that reflect increased maturation. The observed increases in lymphocyte incorporation of ^{3}H-thymidine in PBL and MLN following mitogenic stimulation suggests that adaptive immune function increases with age in these animals in both blood and a tissue in close proximity to the intestine. This was further supported by an increased rate of IL-2 production in stimulated MLN. Lymphocytes from PP did not respond well to the mitogens used in this study, and therefore it was difficult to conclude that adaptive immune function at this site changed during the early post-weaning period. Phenotype data also supports the hypothesis of an increase in adaptive immune function with age. These findings suggest that early weaning, when the piglet adaptive immune system is less well developed, may have negative consequences on the health of the animal.

Table 1. Composition of basal diet.

Ingredient	**Diet (g/kg)**
WHEAT	382
SOYBEAN MEAL	232
CASEIN	232
FAT - Tallow	38.8
- Safflower Oil	21.5
- Linseed Oil	4.70
LIMESTONE	6.00
DICALCIUM PHOSPHATE	18.9
SALT	2.00
SWINE VITAMIN/MINERAL PREMIX[1]	0.750
PC Swine Trace Mineral 3[2]	1.00
Vitamin B12	1.67×10^{-5}
Supplement - Serine	21.0
- Alanine	17.8
- Glycine	15.0
- Sucrose	6.20

[1] The vitamin premix contained (% w/w): 3.8912% Protein, 0.99% Fat, 2.701% Moisture, 86.002 kcal/kg DE, 82.0002 kcal/kg ME, 25.7937% Calcium, 0.225% Phosphorus, 0.0765% Av Phosphorus, 0.1282% Magnesium, 0.0328% Sodium, 642.2313 mg/kg Iron, 7000014.2 IU/kg Vitamin A, 700014.2 IU/kg Vitamin D3, 20000.0117 IU/kg Vitamin E, 1500.0179 mg/kg Vitamin K, 40.0001 mg/kg Biotin, 399.9008 mg/kg Folic Acid, 20000.0117 mg/kg Niacin, 7499.9741 mg/kg Pantothenic Acid, 533.9845 mg/kg Pyridoxine, 3000.0051 mg/kg Riboflavin, 580.5812 mg/kg Thiamine, 10 mg/kg Vitamin B12

[2] The trace mineral premix contained (% w/w): 0.99% fat, 0.001% moisture, 86 kcal/kg DE, 82 kcal/kg ME, 15.1874% Calcium, 0.0729% Magnesium, 0.0283% Sodium, 4.756% Sulfur, 351 mg/kg cobalt, 5000 mg/kg Copper, 749.3 mg/kg Iodine, 75365.1328 mg/kg Iron, 25020 mg/kg Manganese, 150 mg/kg Selenium, 75024 mg/kg Zinc, 100.001 g/kg Choline

Table 2. Neutrophil Burst, Size and Granularity of neutrophils (measure at 21 and 35 days of age)

Measure	Time	Pre-weaning (n=10)	Post-weaning (n=10)
Burst	*0 min.*	24[1,2] ± 10	6 ± 1
	5 min.	98 ± 33	87 ± 13
	fold increase (0-5 min)	10 ± 3	19 ± 3
	10 min.	341 ± 74	358 ± 55
	fold increase (0-10 min)	44 ± 12	75 ± 11
	fold increase (5-10 min)	5 ± 1	4 ± 0.2
	15 min.	752 ± 122	773 ± 103
	fold increase (0-15 min)	122 ± 41	168 ± 30
	fold increase (10-15 min)	3 ± 0.4	2 ± 0.2
Size	*0 min.*	499 ± 21	503 ± 17
	5 min.	533 ± 23	531 ± 19
	10 min.	565 ± 19	565 ± 17
	15 min.	572 ± 14	569 ± 13
Granularity	*0 min.*	307 ± 31	280 ± 7
	5 min.	339 ± 40	291 ± 5
	10 min.	331 ± 34	292 ± 4
	15 min.	333 ± 40	300 ± 5

[1] Values presented as mean fluorescence ± SEM

[2] There were no significant differences ($p<0.05$) in any measure of neutrophil function both prior to (0 min) or after stimulation with PMA

Table 3. Cell Phenotyping from PBL at 21 and 35 days of Age

Antibody	Pre-weaning (n=9)	Post-weaning (n=10)
IGA	12[1] ± 4	9 ± 1
IGM	13 ± 2	16 ± 1
CD44+BCELL+	17 ± 2	18 ± 3
% B cells CD44+	89^{a} ± 2	90^{b} ± 3
CD44+CD3+	30^{a} ± 9	44^{b} ± 13
% CD3 CD44+	97 ± 2	97 ± 1
CD4+CD45RA+	29 ± 5	29 ± 5
CD4+CD45RA-	17 ± 3	12 ± 2
CD8+CD45RA+	28 ± 5	23 ± 2
CD8+CD45RA-	9 ± 2	5 ± 1
CD2+CD3+	55^{b} ± 5	28^{a} ± 5
CD2+CD3-[3]	20 ± 6	14 ± 3
CD3+CD4+	30 ± 7	41 ± 5
CD3+CD8+	29 ± 6	23 ± 5
CD3+	66^{b} ± 4	54^{a} ± 4
CD4+	34 ± 5	50 ± 4
CD8+	35 ± 4	29 ± 3
CD4/CD8	1.0 ± 0.2	1.9 ± 0.3
CD25+	25 ± 8	24 ± 2
Ra+	60 ± 6	66 ± 3
CD44+	95 ± 1	95 ± 1
Monocyte+	17 ± 2	16 ± 1
B cell+	22 ± 2	24 ± 2
CD2+	72^{b} ± 6	54^{a} ± 9

[1] Values presented as cell population percentage mean ± SEM

[2] Means within a row that do not share a common letter are significantly different ($p<0.05$)

[3] This population was assumed to be the CD4CD8 double negative T cell subset

Table 4. Cell Phenotyping from MLN Lymphocytes from piglets at 21 and 35 days of Age

Antibody	Pre-weaning (n=9)	Post-weaning (n=10)
IGA	10[a][1,2] ± 2	17[b] ± 1
IGM	23 ± 3	22 ± 2
CD4+CD45RA+	30 ± 6	41 ± 4
CD4+CD45RA-	17 ± 3	13 ± 2
CD8+CD45RA+	27 ± 2	30 ± 2
CD8+CD45RA-	8 ± 2	6 ± 1
CD4+CD25+	20 ± 4	15 ± 2
CD8+CD25+	24 ± 2	16 ± 3
CD2+CD3+	31 ± 7	33 ± 3
CD2+CD3-[3]	21 ± 7	28 ± 6
CD3+	44 ± 4	51 ± 2
CD4+	48 ± 3	49 ± 2
CD8+	38 ± 1	38 ± 3
CD4/CD8	1.3 ± 0.1	1.4 ± 0.1
CD25+	30 ± 4	22 ± 3
Ra+	71[a] ± 6	77[b] ± 2
B cell+	28[a] ± 4	39[b] ± 4
CD2+	52 ± 9	61 ± 8

[1] Values presented as cell population percentage mean ± SEM

[2] Means within a row that do not share a common letter are significantly different ($p<0.05$)

[3] This population was assumed to be the CD4CD8 double negative T cell subset

Table 5. Cell Phenotyping from PP at 21 and 35 days of Age

Antibody	Pre-weaning (n=7)	Post-weaning (n=10)
IGA	$9^{1} \pm 3$	5 ± 1
IGM	41 ± 5	40 ± 3
PP BCELL	$9^{2a} \pm 1$	$20^{b} \pm 4$
CD44+BCELL+	12 ± 3	9 ± 2
% B cell CD44+	21 ± 4	27 ± 7
CD4+CD45RA+	9 ± 1	17 ± 7
CD4+CD45RA-	$4^{a} \pm 1$	$5^{b} \pm 1$
CD8+CD45RA+	10 ± 1	14 ± 3
CD8+CD45RA-	5 ± 1	7 ± 3
CD3+	$14^{a} \pm 4$	$25^{b} \pm 3$
CD4+	13 ± 1	22 ± 7
CD8+	15 ± 1	21 ± 6
CD4/CD8	0.9 ± 0.1	1.5 ± 0.6
RA+	$89^{b} \pm 4$	$82^{a} \pm 4$
CD44+	29 ± 5	31 ± 6
Monocyte+	23 ± 3	21 ± 3
B cell+	53 ± 2	46 ± 4
CD2+	25 ± 6	33 ± 3

[1] Values presented as cell population percentage mean ± SEM

[2] Means within a row that do not share a common letter are significantly different ($p<0.05$)

Table 6. ^{3}H-Thymidine Uptake by PBL, MLN and PP at 21 and 35 days of Age

Tissue (Mitogen)		Pre-weaning (n=10)	Post-weaning (n=10)
PBL (none)	**DPM**[1]	3921^{b}[2] ± 829	1036^{a} ± 429
(PHA)	**DPM**	173292^{a} ± 42454	287271^{b} ± 47681
Log transformed[3]	**SI**[4]	1.7[2]^{a} ± 0.1	2.6^{b} ± 0.1
(PWM)	**DPM**	104660^{a} ± 22079	162828^{b} ± 28855
Log transformed	**SI**	1.5^{a} ± 0.1	2.3^{b} ± 0.2
(LPS)	**DPM**	10031^{b} ± 1725	2900^{a} ± 907
Log transformed	**SI**	0.4 ± 0.2	0.2 ± 0.4
MLN (none)	**DPM**	176 ± 35	236 ± 30
(PHA)	**DPM**	168208^{a} ± 39434	291989^{b} ± 41485
	SI	1021 ± 198	1745 ± 457
(PWM)	**DPM**	111266^{a} ± 25512	203420^{b} ± 28835
	SI	649^{a} ± 126	1130^{b} ± 254
(LPS)	**DPM**	178^{a} ± 42	391^{b} ± 86
	SI	0.4 ± 0.3	0.8 ± 0.2
PP (none)	**DPM**	96 ± 22	67 ± 11
(PHA) Log transformed	**DPM**	3.0 ± 0.3	2.7 ± 0.1
Log transformed	**SI**	1.0 ± 0.4	0.8 ± 0.1
(PWM) Log transformed	**DPM**	2.7 ± 0.3	2.3 ± 0.1
Log transformed	**SI**	0.5 ± 0.5	0.3 ± 0.2
(LPS)	**DPM**	83 ± 20	72 ± 18
	SI	0.4 ± 0.3	0.2 ± 0.1

[1] Values reported in decays per minute (mean ± SEM)

[2] Means within a row that do not share a common letter are significantly different ($p<0.05$)

[3] Kurtosis and Skew were analyzed for proliferation data and values that were not considered normally distributed were log transformed.

[4] Values presented as mean stimulation index ± SEM, calculated as DPM stimulated/DPM unstimulated

Table 7. Cytokine Production by piglet PBL taken at 21 and 35 days of Age to PHA

Cytokine	Pre-weaning (n=10)	Post-weaning (n=10)
IL-4 (Log transformed)[1]	1.5[2] ± 0.1	1.7 ± 0.1
IL-2	1478 ± 495	713 ± 273
IFN-γ	902 ± 271	406 ± 140
TNF-α (Log transformed)	1.5 ± 0.03	1.5 ± 0.04
IL-10 (Log transformed)	1.2 ± 0.1	1.1 ± 0.2
IFN-γ/IL-4 (Log transformed)	1.3^{b}[3] ± 0.1	0.6^{a} ± 0.1

[1] Kurtosis and Skew were analyzed for cytokine data and values that were not considered normally distributed were log transformed.

[2] Values presented as mean ± SEM

[3] Means within a row that do not share a common letter are significantly different ($p<0.05$)

Table 8. Cytokine Production by piglet MLN Lymphocytes taken at 21 and 35 days of Age to PHA

Cytokine	Pre-weaning (n=10)	Post-weaning (n=10)
IL-4	226[1] ± 29	259 ± 36
IL-2 (Log transformed)[2]	2.4^{a}[3] ± 0.2	2.9^{b} ± 0.1
IFN-γ	269 ± 77	365 ± 96
TNF-α	28 ± 1	32 ± 2
IL-10	74 ± 10	58 ± 12
IFN-γ/IL-4 (Log transformed)	-0.1 ± 0.1	0.05 ± 0.1

[1] Values presented as mean ± SEM

[2] Kurtosis and Skew were analyzed for cytokine data and values that were not considered normally distributed were log transformed.

[3] Means within a row that do not share a common letter are significantly different ($p<0.05$)

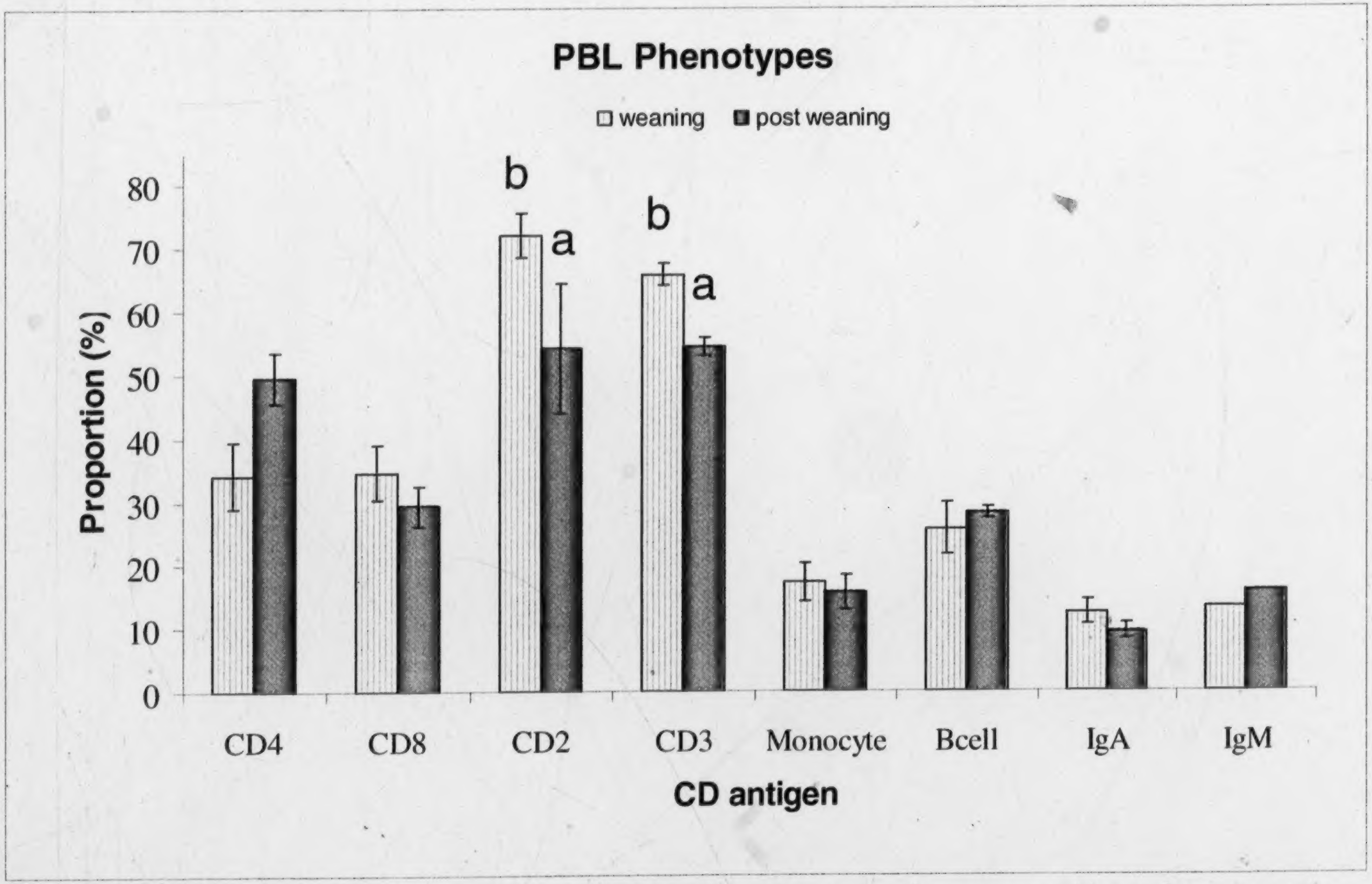


Figure 1. PBL phenotypes obtained from piglets at 21 and 35 days of age. Phenotypes given as mean with error bars representing SEM. Phenotypes that do not share a common letter are significantly different ($p<0.05$).

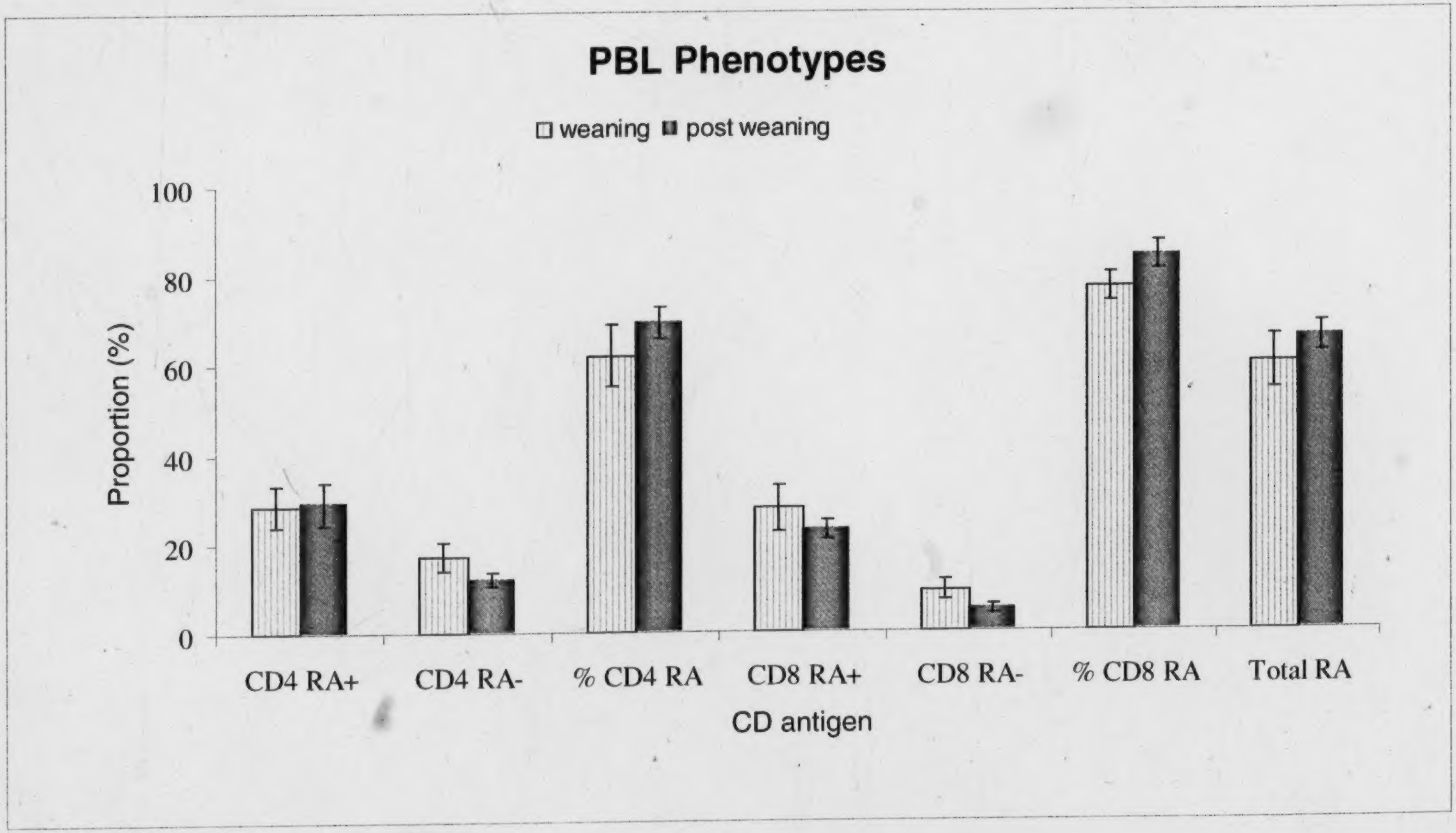


Figure 2. PBL phenotypes with percentage of T cell expressing CD45RA antigen. Phenotypes given as mean with error bars representing SEM. CD45RA phenotypes were not significantly different (p<0.05).

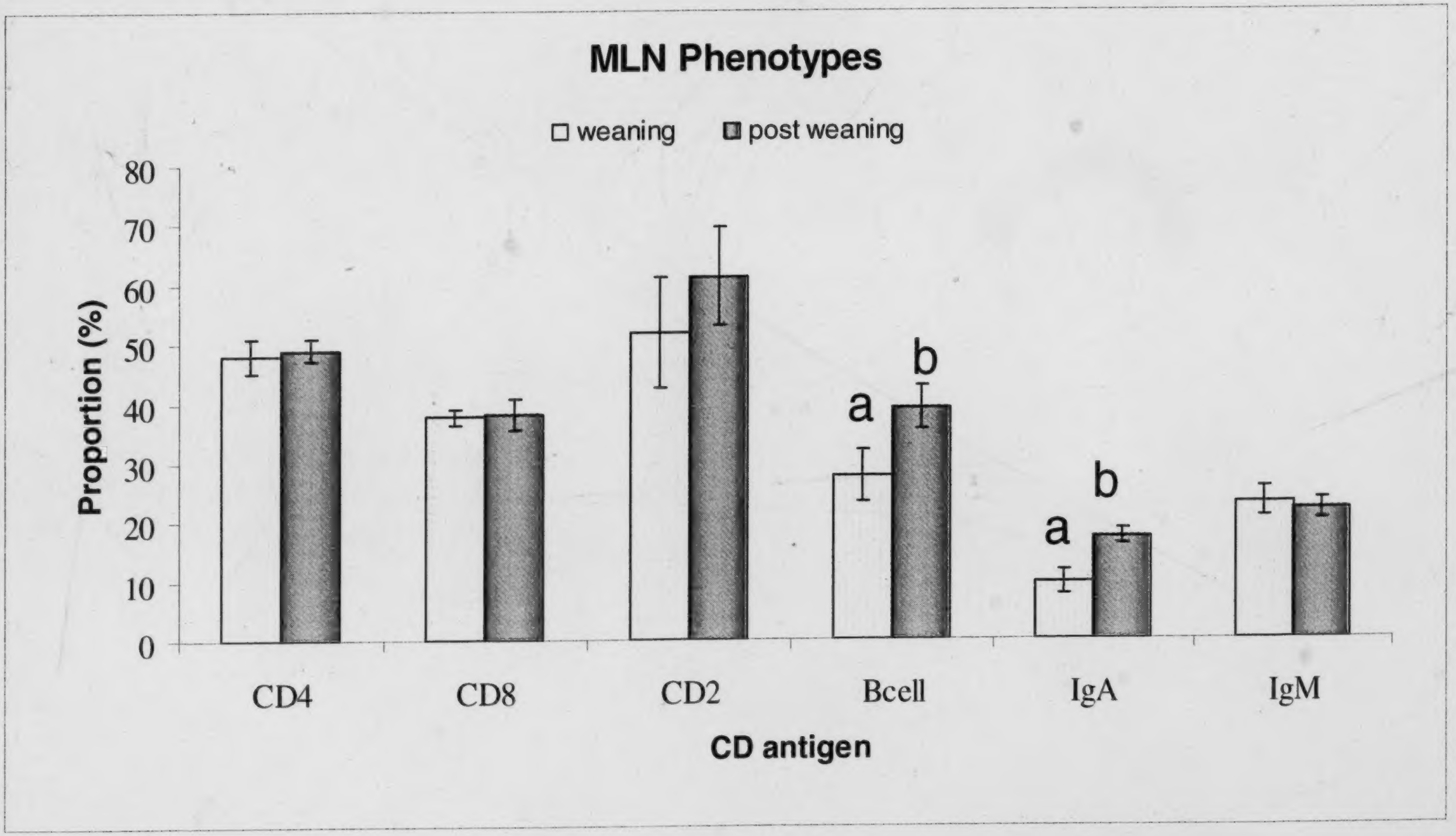


Figure 3. MLN phenotypes obtained from piglets at 21 and 35 days of age. Phenotypes given as mean with error bars representing SEM. Phenotypes that do not share a common letter are significantly different ($p<0.05$).

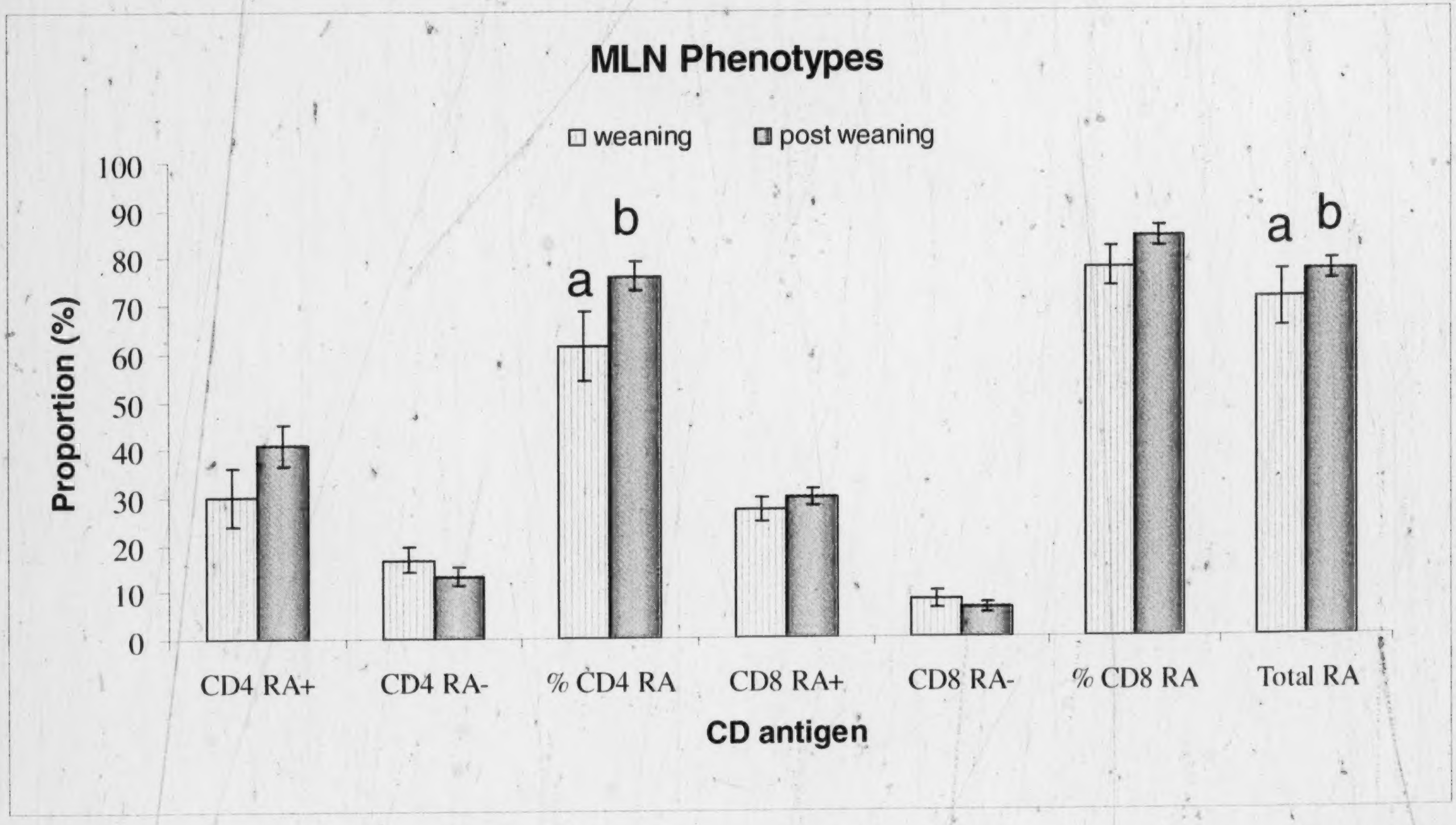


Figure 4. MLN phenotypes with percentage of T cells expressing CD45RA antigen. Phenotypes given as mean with error bars representing SEM. Phenotypes that do not share a common letter are significantly different ($p<0.05$).

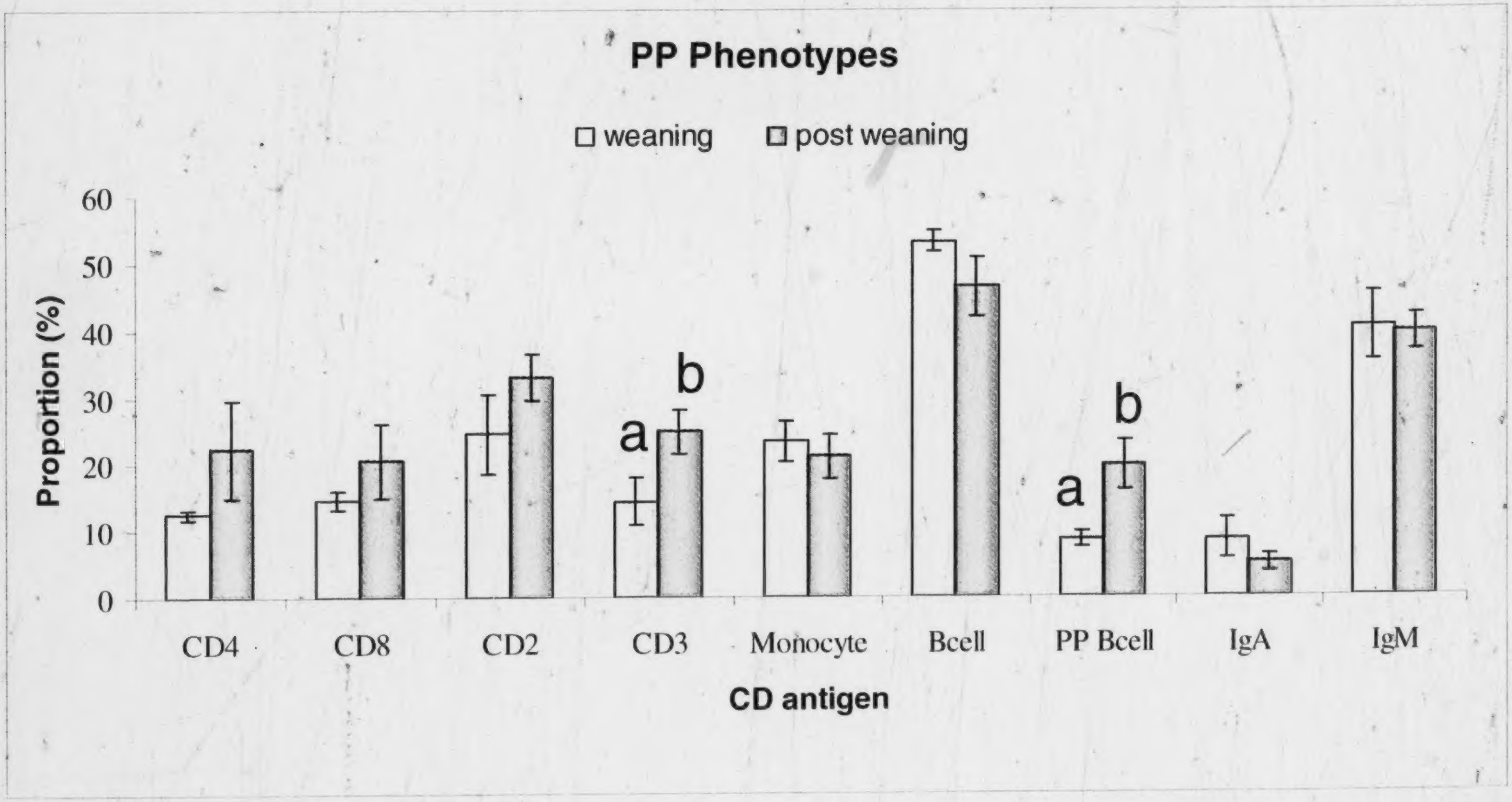


Figure 5. PP phenotypes obtained from piglets at 21 and 35 days of age. Phenotypes given as mean with error bars representing SEM. Phenotypes that do not share a common letter are significantly different ($p<0.05$).

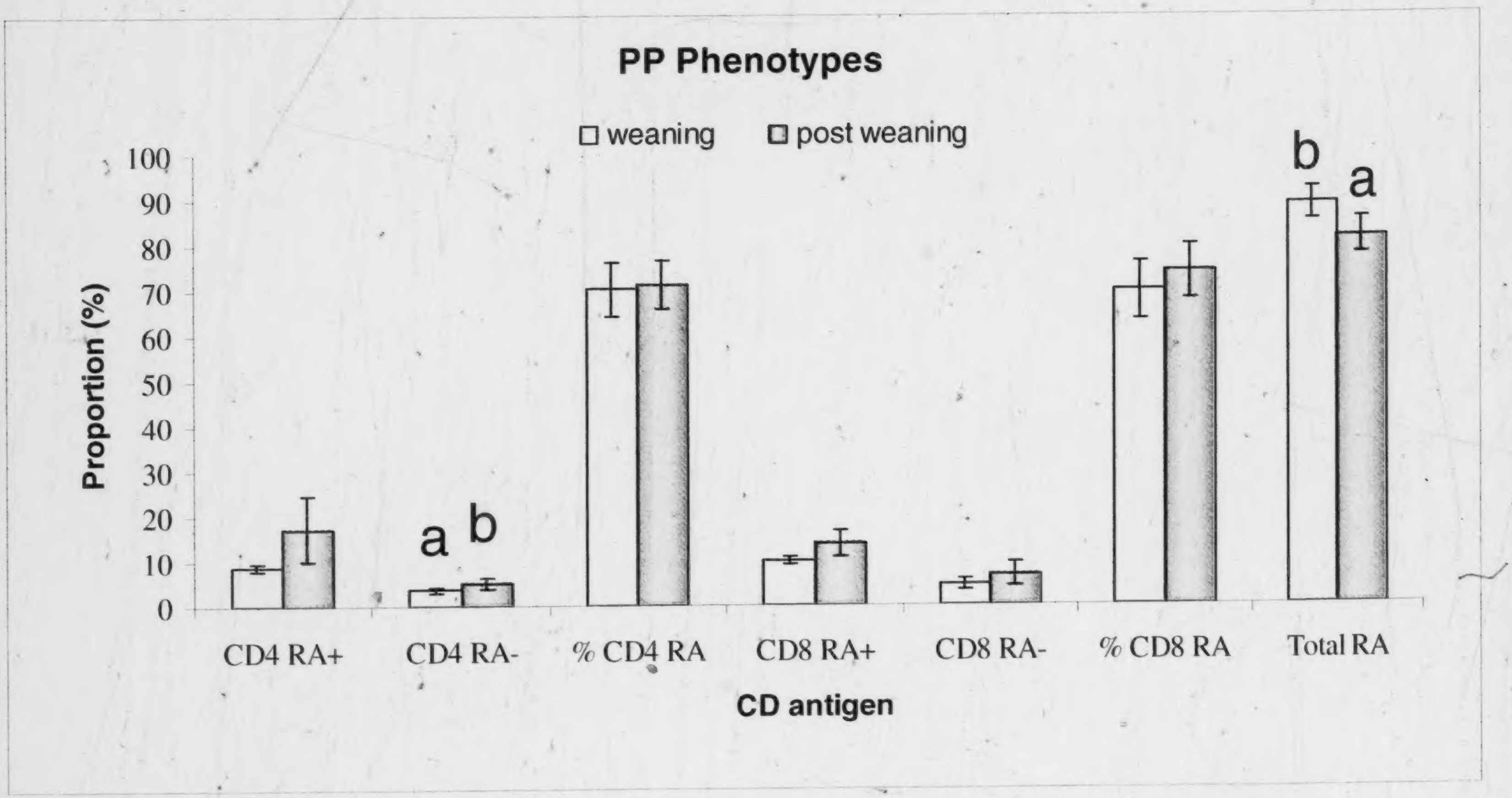


Figure 6. PP phenotypes with percentage of T cells expressing CD45RA antigen. Phenotypes given as mean with error bars representing SEM. Phenotypes that do not share a common letter are significantly different ($p<0.05$).

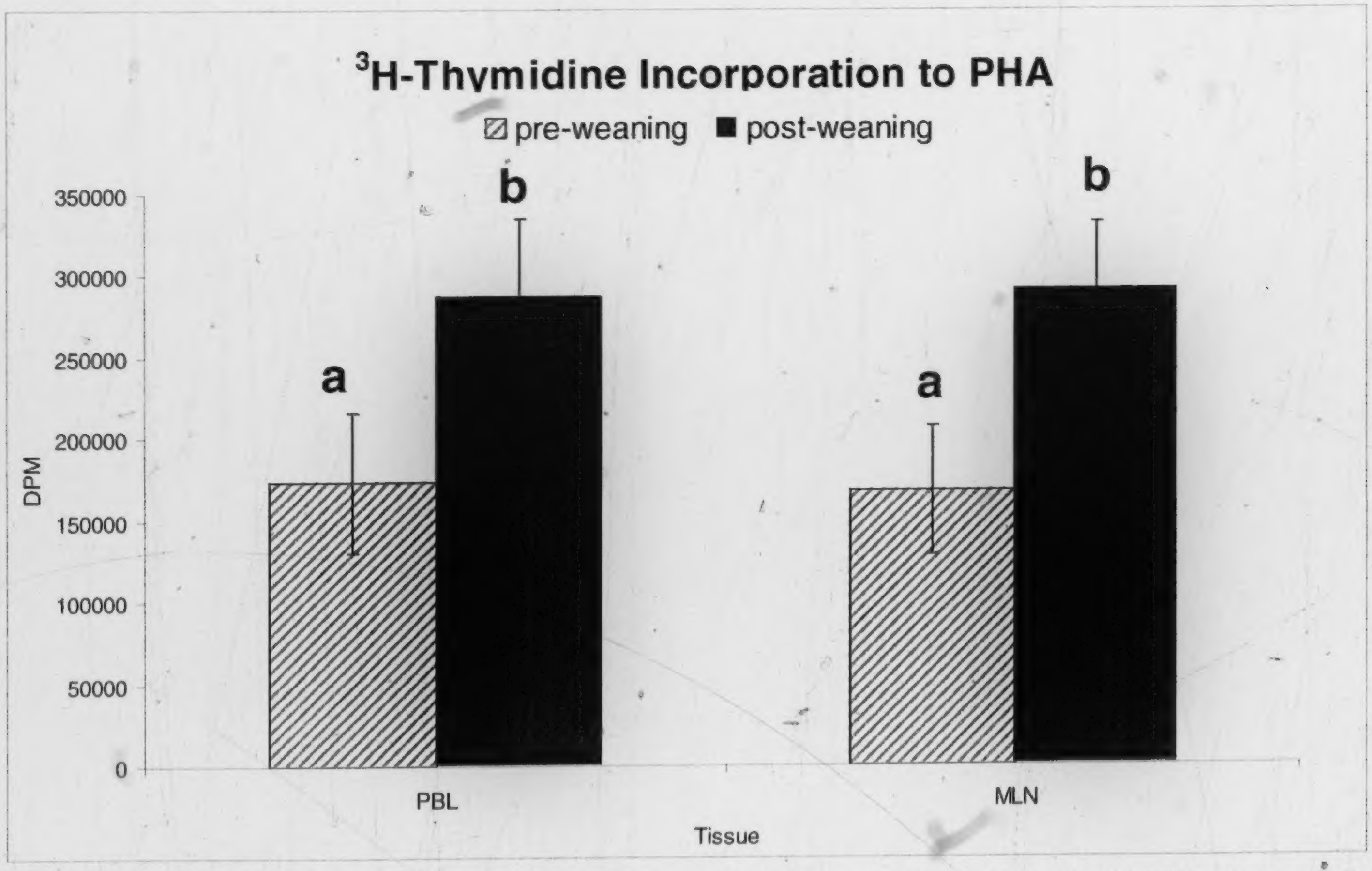


Figure 7. Proliferation of PBL and MLN lymphocytes from piglets at 21 and 35 days of age stimulated with PHA mitogen. Indicated as mean Decays per Minute (DPM) with error bars representing SEM. DPMs that do not share a common letter are significantly different ($p<0.05$).

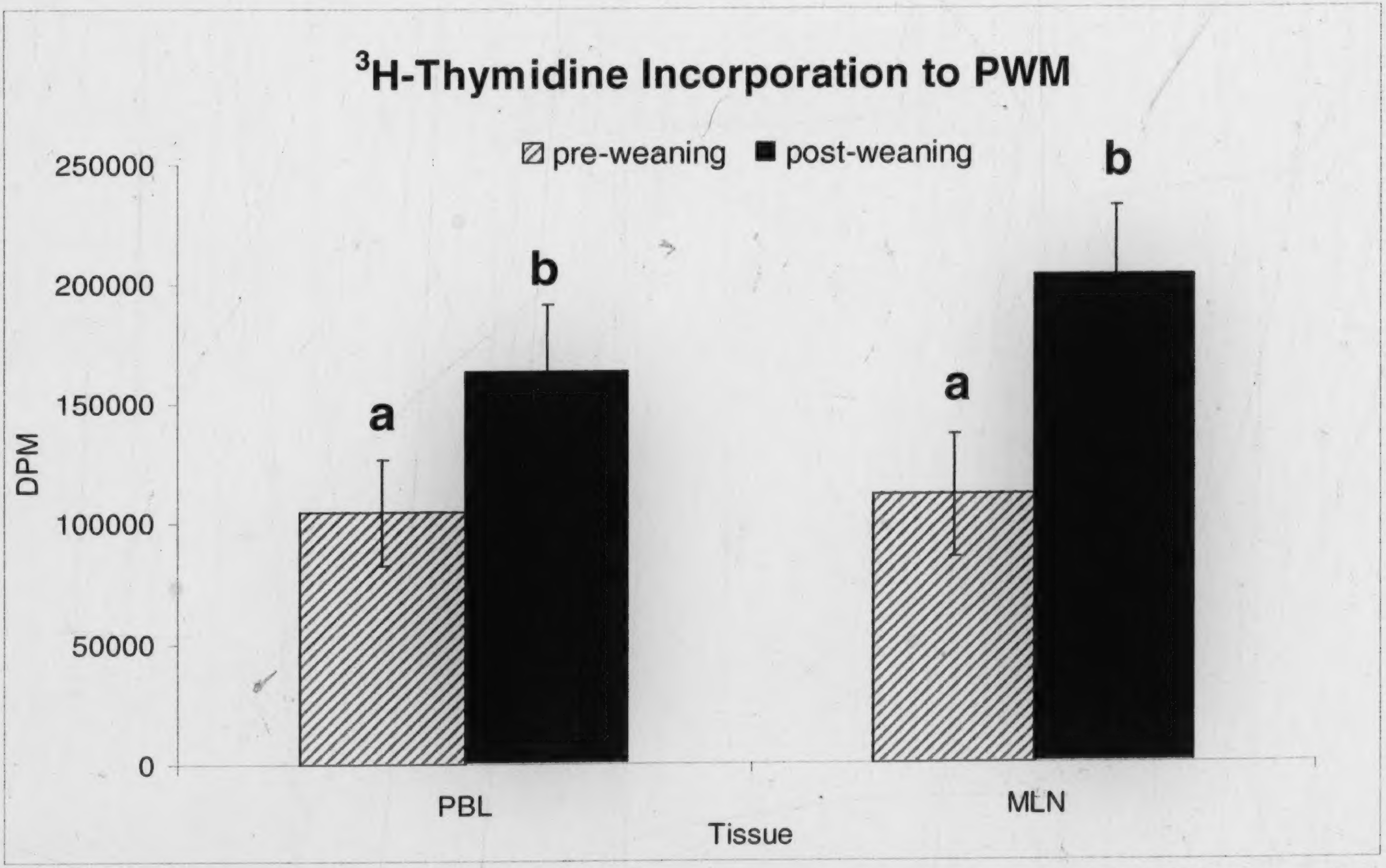


Figure 8. Proliferation of PBL and MLN lymphocytes from piglets at 21 and 35 days of age stimulated with PWM mitogen. Indicated in mean decays per minute (DPM) with error bars representing SEM. DPMs that do not share a common letter are significantly different ($p<0.05$).

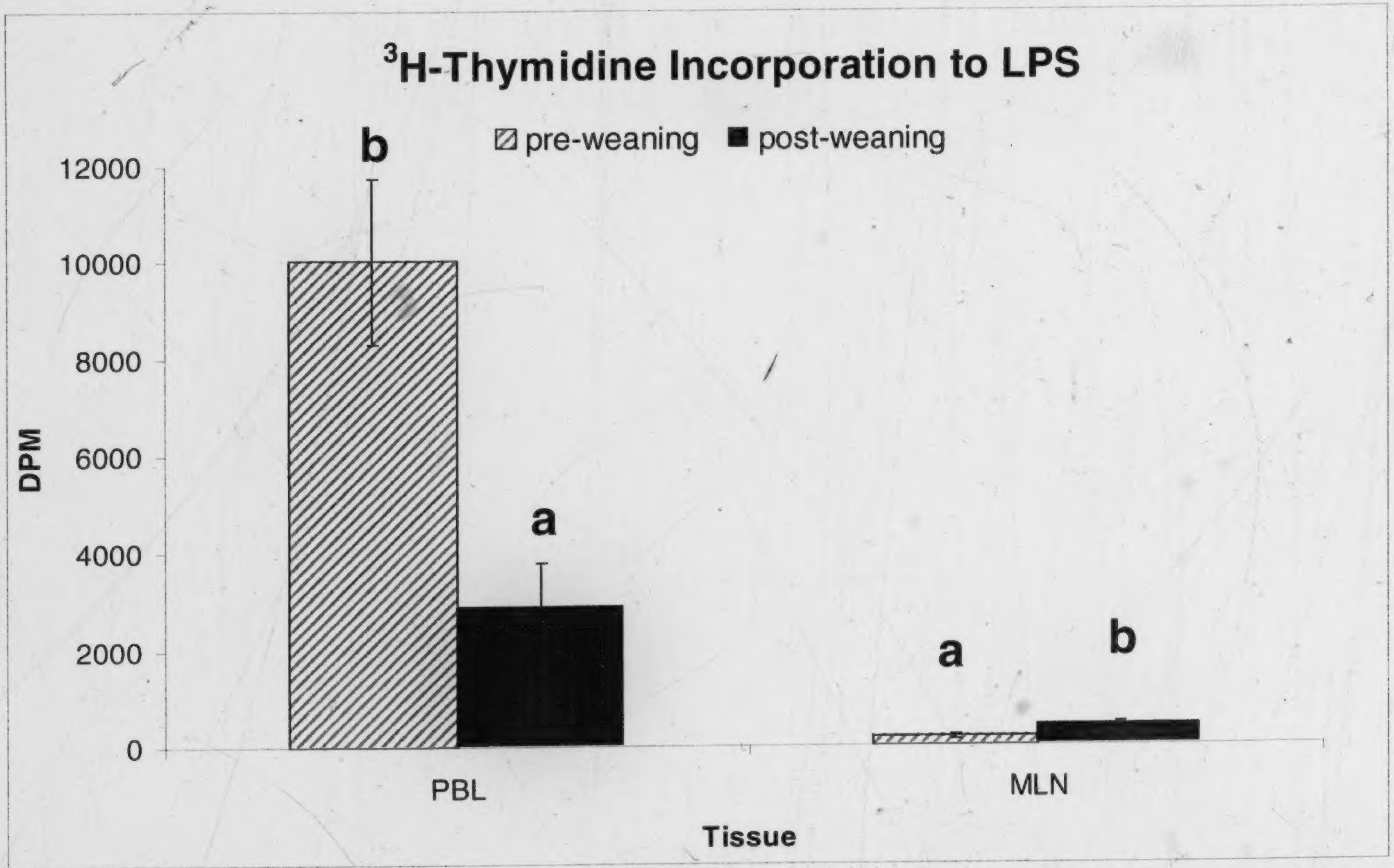


Figure 9. Proliferation of PBL and MLN lymphocytes from piglets at 21 and 35 days of age stimulated with LPS mitogen. Indicated as mean decays per minute (DPM) with error bars representing SEM. DPMs that do not share a common letter are significantly different ($p<0.05$).

4.5 REFERENCES

Arriens,M.A., Summerfield,A., and McCullough,K.C. 1998. Differential adhesion molecule expression on porcine mononuclear cell populations. Scand. J. Immunol. 47: 487-495.

Bailey,M., Hall,L., Bland,P.W., and Stokes,C.R. 1994. Production of cytokines by lymphocytes from spleen, mesenteric lymph node and intestinal lamina propria of pigs. Immunology 82: 577-583.

Bailey,M., Plunkett,F., Clarke,A., Sturgess,D., Haverson,K., and Stokes,C.R. 1998. Activation of T cells from the intestinal lamina propria of the pig. Scand. J. Immunol. 48: 177-182.

Bailey,M., Plunkett,F.J., Rothkötter,H.J., Vega-Lopez,M.A., Haverson,K., and Stokes,C.R. 2001. Regulation of mucosal immune responses in effector sites. Proc. Nutr. Soc. 60: 427-435.

Barnett,K.L., Kornegay,E.T., Risley,C.R., Lindemann,M.D., and Schurig,G.G. 1989. Characterization of creep feed consumption and its subsequent effects on immune response, scouring index and performance of weanling pigs. J. Anim Sci. 67: 2698-2708.

Becker,B.A. and Misfeldt,M.L. 1993. Evaluation of the mitogen-induced proliferation and cell surface differentiation antigens of lymphocytes from pigs 1 to 30 days of age. J. Anim Sci. 71: 2073-2078.

Bianchi,A.T., Zwart,R.J., Jeurissen,S.H., and Moonen-Leusen,H.W. 1992. Development of the B- and T-cell compartments in porcine lymphoid organs from birth to adult life: an immunohistological approach. Vet. Immunol. Immunopathol. 33: 201-221.

Blecha,F., Pollmann,D.S., and Nichols,D.A. 1983. Weaning pigs at an early age decreases cellular immunity. J. Anim Sci. 56: 396-400.

Braakman,E., Sturm,E., Vijverberg,K., van Krimpen,B.A., Gratama,J.W., and Bolhuis,R.L. 1991. Expression of CD45 isoforms by fresh and activated human gamma delta T lymphocytes and natural killer cells. Int. Immunol. **3:** 691-697.

Camp,R.L., Kraus,T.A., Birkeland,M.L., and Pure,E. 1991. High levels of CD44 expression distinguish virgin from antigen-primed B cells. J. Exp. Med. 173: 763-766.

Choi,I.S. and Yoo,H.S. 2002. Expression of recombinant porcine interleukin-2 and application of its antibody to immunoassays. J. Vet. Sci. 3: 207-212.

Chu,R.M., Glock,R.D., Ross,R.F., and Cox,D.F. 1979. Lymphoid tissues of the small intestine of swine from birth to one month of age. Am. J. Vet. Res. 40: 1713-1719.

Denham,S., Zwart,R.J., Whittall,J.T., Pampusch,M., Corteyn,A.H., Bianchi,A.T., Murtaugh,M.P., Parkhouse,R.M., Tlaskalova,H., Sinkora,J., Sinkora,M., and Rehakova,Z. 1998. Monoclonal antibodies putatively identifying porcine B cells. Vet. Immunol. Immunopathol. 60: 317-328.

Dréau,D., Lallès,J.P., Philouze-Romé,V., Toullec,R., and Salmon,H. 1994. Local and systemic immune responses to soybean protein ingestion in early-weaned pigs. J. Anim. Sci. 72: 2090-2098.

Field,C.J., Clandinin,M.T., and Van Aerde,J.E. 2001. Polyunsaturated fatty acids and T-cell function: implications for the neonate. Lipids 36: 1025-1032.

Friesen,K.G., Goodband,R.D., Nelssen,J.L., Blecha,F., Reddy,D.N., Reddy,P.G., and Kats,L.J. 1993. The effect of pre- and postweaning exposure to soybean meal on the growth performance and on the immune response in the early-weaned pig. J. Anim. Sci. 71: 2089-2098.

Haks,M.C., Oosterwegel,M.A., Blom,B., Spits,H.M., and Kruisbeek,A.M. 1999. Cell-fate decisions in early T cell development: regulation by cytokine receptors and the pre-TCR. Semin. Immunol. 11: 23-37.

Hampson,D.J. 1986. Attempts to modify changes in the piglet small intestine after weaning. Res. Vet. Sci. 40: 313-317.

Hoskinson,C.D., Chew,B.P., and Wong,T.S. 1990. Age-related changes in mitogen-induced lymphocyte proliferation and polymorphonuclear neutrophil function in the piglet. J. Anim Sci. 68: 2471-2478.

Insoft,R.M., Sanderson,I.R., and Walker,W.A. 1996. Development of immune function in the intestine and its role in neonatal diseases. Pediatr. Clin. North Am. 43: 551-571.

Jonjic,N., Jonjic,S., Saalmuller,A., Rukavina,D., and Koszinowski,U.H. 1987. Distribution of T-lymphocyte subsets in porcine lymphoid tissues. Immunology 60: 395-401.

Keren,D.F. 1992. Antigen processing in the mucosal immune system. Semin. Immunol. 4: 217-226.

Kirkham,P.A., Takamatsu,H., Yang,H., and Parkhouse,R.M. 1996. Porcine CD3 epsilon: its characterization, expression and involvement in activation of porcine T lymphocytes. Immunology 87: 616-623.

Lawrence,K.R., Hastad,C.W., Goodband,R.D., Tokach,M.D., Dritz,S.S., Nelssen,J.L., DeRouchey,J.M., and Webster,M.J. 2003. Effects of soybean meal particle size on growth performance of nursery pigs. J. Anim. Sci. 81: 2118-1122.

Li,D.F., Nelssen,J.L., Reddy,P.G., Blecha,F., Hancock,J.D., Allee,G.L., Goodband,R.D., and Klemm,R.D. 1990. Transient hypersensitivity to soybean meal in the early-weaned pig. J. Anim. Sci. 68: 1790-1799.

Lunney,J.K. and Pescovitz,M.D. 1987. Phenotypic and functional characterization of pig lymphocyte populations. Vet. Immunol. Immunopathol. 17: 135-144.

Miyake,K. 2003. Innate recognition of lipopolysaccharide by CD14 and toll-like receptor 4-MD-2: unique roles for MD-2. Int. Immunopharm. 3: 119-128.

Murakami,S., Miyake,K., June,C.H., Kincade,P.W., and Hodes,R.J. 1990. IL-5 induces a Pgp-1 (CD44) bright B cell subpopulation that is highly enriched in proliferative and Ig secretory activity and binds to hyaluronate. J. Immunol. 145: 3618-3627.

Nabuurs,M.J. 1998. Weaning piglets as a model for studying pathophysiology of diarrhea. Vet. Q. 20 Suppl 3: S42-S45.

NRC 1998. Nutrient Requirements of swine (10th Ed.). Subcom. Swine Nutr., Comm Anim. Nutr., Board Agric., NRC. Nat. Acad. Press, Wash. pp 192.

Pabst,R. and Rothkotter,H.J. 1999. Postnatal development of lymphocyte subsets in different compartments of the small intestine of piglets. Vet. Immunol. Immunopathol. 72: 167-173.

Patience,J.F. and Thacker,P.A. 1989. Swine Nutrition Guide. Univ. Saskat. and Prairie Swine Ctr, Saskatoon, Sask. pp 260.

Pescovitz,M.D., Hsu,S.M., Katz,S.I., Lunney,J.K., Shimada,S., and Sachs,D.H. 1990. Characterization of a porcine CD1-specific mAb that distinguishes CD4/CD8 double-positive thymic from peripheral T lymphocytes. Tissue Antigens 35: 151-156.

Pescovitz,M.D., Sakopoulos,A.G., Gaddy,J.A., Husmann,R.J., and Zuckermann,F.A. 1994. Porcine peripheral blood CD4+/CD8+ dual expressing T-cells. Vet. Immunol. Immunopathol. 43: 53-62.

Rodriguez-Carreno,M.P., Lopez-Fuertes,L., Revilla,C., Ezquerra,A., Alonso,F., and Dominguez,J. 2002. Phenotypic characterization of porcine IFN-gamma-producing lymphocytes by flow cytometry. J. Immunol. Methods 259: 171-179.

Rothkotter,H.J. and Pabst,R. 1989. Lymphocyte subsets in jejunal and ileal Peyer's patches of normal and gnotobiotic minipigs. Immunology 67: 103-108.

Saalmuller,A., Jonjic,S., Buhring,H.J., Reddehase,M.J., and Koszinowski,U.H. 1987a. Monoclonal antibodies reactive with swine lymphocytes. II. Detection of an antigen on resting T cells down-regulated after activation. J. Immunol. 138: 1852-1857.

Saalmuller,A., Reddehase,M.J., Buhring,H.J., Jonjic,S., and Koszinowski,U.H. 1987b. Simultaneous expression of CD4 and CD8 antigens by a substantial proportion of resting porcine T lymphocytes. Eur. J. Immunol. 17: 1297-1301.

Saalmuller,A., Hirt,W., and Reddehase,M.J. 1989. Phenotypic discrimination between thymic and extrathymic CD4-CD8- and CD4+CD8+ porcine T lymphocytes. Eur. J. Immunol. 19: 2011-2016.

Saalmuller,A. and Bryant,J. 1994. Characteristics of porcine T lymphocytes and T-cell lines. Vet. Immunol. Immunopathol. 43: 45-52.

Sanders,M.E., Makgoba,M.W., Sharrow,S.O., Stephany,D., Springer,T.A., Young,H.A., and Shaw,S. 1988. Human memory T lymphocytes express increased levels of three cell adhesion molecules (LFA-3, CD2, and LFA-1) and three other molecules (UCHL1, CDw29, and Pgp-1) and have enhanced IFN-gamma production. J. Immunol. 140: 1401-1407.

Shivtiel,S., Leider,N., and Melamed,D. 2002. Receptor editing in CD45-deficient immature B cells. Eur. J. Immunol. 32: 2264-2273.

Solano-Aguilar,G.I., Vengroski,K.G., Beshah,E., Douglass,L.W., and Lunney,J.K. 2001. Characterization of lymphocyte subsets from mucosal tissues in neonatal swine. Dev. Comp Immunol. 25: 245-263.

Stokes,C.R., Haverson,K., and Bailey,M. 1996. Antigen presenting cells in the porcine gut. Vet. Immunol. Immunopathol. 54: 171-177.

Summerfield,A., Rziha,H.J., and Saalmuller,A. 1996. Functional characterization of porcine CD4+CD8+ extrathymic T lymphocytes. Cell Immunol. 168: 291-296.

Valpotic,I., Gerencer,M., and Basic,I. 1989. In vitro modulating effects of porcine immunoglobulin G on mitogens-induced lymphocyte response in precolostral, suckling and weaned piglets. Vet. Immunol. Immunopathol. 22: 113-122.

Vega-Lopez,M.A., Bailey,M., Telemo,E., and Stokes,C.R. 1995. Effect of early weaning on the development of immune cells in the pig small intestine. Vet. Immunol. Immunopathol. 44: 319-327.

Whary,M.T., Zarkower,A., Confer,F.L., and Ferguson,F.G. 1995. Age-related differences in subset composition and activation responses of intestinal intraepithelial and mesenteric lymph node lymphocytes from neonatal swine. Cell Immunol. 163: 215-221.

Yang,H. and Binns,R.M. 1993. Expression and regulation of the porcine CD44 molecule. Cell Immunol. 149: 117-129.

Zuckermann,F.A. and Gaskins,H.R. 1996. Distribution of porcine CD4/CD8 double-positive T lymphocytes in mucosa-associated lymphoid tissues. Immunology 87: 493-499.

Chapter 5 – INFLUENCE OF GLUTAMINE ON IMMUNE AND GUT DEVELOPMENT AND ON THE EARLY STAGES OF ETEC INFECTION IN NEWLY WEANED PIGLETS

5.1 INTRODUCTION

Early weaning results in adverse reactions of the piglet, and diarrhea is the first and most prevalent clinical sign of illness after early weaning (Nabuurs 1998). Dietary intervention aimed at improving the development of the immune system so it can aid in the prevention of ETEC would be a very effective method of helping reduce the risk of infection in piglets. The importance of gln during infections has been demonstrated *in vivo* by its ability to preserve epithelial barrier function in the distal ileum of 21-d old piglets (Dugan & McBurney 1995), significantly decrease the mean duration of diarrhea in children (Yalcin et al. 2004), and in rodents to lower translocation rates of radiolabeled *E. coli* (Gennari & Alexander 1997), improve survival (Gennari & Alexander 1997; Suzuki et al. 1993) and pathogen elimination (Suzuki et al. 1993). *In vitro*, gln is also important for the maintenance of epithelial barrier function, demonstrated in Caco-2 cells (Panigrahi et al. 1997; DeMarco et al. 2003). Pathogens such as ETEC influence intestinal permeability of tight junctions and was shown to decrease the number of tight junction strands in three-week old piglets (Egberts et al. 1993). The mechanism appears to involve intracellular increases in cyclic 3^1,5^1-adenosine monophosphate (cAMP), which is induced by ETEC and has been shown *in vitro* to cause either a breakdown or proliferation of interepithelial tight junctions (Duffey et al. 1981).

The potential increased requirement for gln, and it's classification as being "conditionally essential", is demonstrated by the finding that both immune function and plasma gln concentrations are depressed in a variety of "stress" conditions (reviewed in (Field et al. 2000a). *In vitro*, Gln is an energy substrate for lymphocytes and lymphocyte proliferation, B lymphocyte (B-cell) differentiation, and cytokine production IL-1, IL-2, IFN-γ and TNF-α) (reviewed in (Newsholme & Parry-Billings 1990; Newsholme & Calder 1997)). Provision of enteral gln has also been shown to increase T lymphocyte (T cell) function and positively influence T cell proportions (Jensen et al. 1996).

Gln is important in innate immune defense, as evidenced that it is also an energy substrate for macrophages (reviewed in (Newsholme & Parry-Billings 1990)). There is also data to support the importance of gln for *in vitro* neutrophil bactericidal function (Ogle et al. 1994). *In vitro*, gln has also been shown to be involved in the production of nitric oxide (NO) (Murphy & Newsholme 1998; O'Dowd & Newsholme 1997) and IL-1 (reviewed in (Newsholme & Calder 1997)) by macrophages. Increases of glycyl gln concentrations *in vitro* significantly increased antimicrobial functions (generation of superoxide and killing capacity) of human monocytes (Ruggeberg et al. 1997). Gln is also known to facilitate phagocytosis (reviewed in (Newsholme et al. 1985; Parry-Billings et al. 1990; van Acker et al. 1999)). Polymorphonuclear neutrophils (neutrophils) contribute to host defense by migrating to sites of infection and trauma and upon encounter with bacteria, activates its diverse oxygen-dependent (reactive oxygen intermediates (ROI)) and oxygen-independent (phagocytosis of opsonized foreign bodies and release of other microbicidal substances) antimicrobial systems by releasing azurophil and specific granules to the phagosome or the exterior of the cell (Chertov et al. 2000; Faurschou & Borregaard 2003). Most of the steps in the elimination of foreign microorganisms are dependent on the mobilization of cytoplasmic granules and secretory vesicles (Faurschou & Borregaard 2003). Neutrophil granule proteins, due to their proximal location and rapid release, are major components in innate host defense and play potentially pivotal roles in the initiation of adaptive immune responses to bacteria, viruses and fungi (Chertov et al. 2000).

Gln has also been shown to influence intestinal morphology. Provision of gln by total parenteral nutrition has been shown to preserve mucosal gln (van der Hulst et al. 1996) and glutathione levels (Basoglu et al. 1997). It was demonstrated to be a key nutrient in the maintenance of the gut mucosa; addition of gln to TPN solutions (reviewed in (Alverdy 1990)) and in the diet of newly weaned piglets (Wu 1996) partially prevented intestinal atrophy. When provided orally to rodents, gln was implicated in preserving intestinal metabolism, structure and function by accelerating healing of the gut mucosa in irradiated animals by supporting mitosis in the proliferative zone of the villous crypts (Klimberg et al. 1990).

Despite the important roles attributed to gln, supplementation to piglets has received little attention (Yoo et al. 1997). The objective of this study was to determine the ability of supplementing the diet with additional gln (4.4 % w/w) to promote immune function and defense against ETEC challenge and to improve gastrointestinal function against an ETEC challenge in the early post-weaning period in piglets. It was hypothesized that supplementation of dietary gln to newly weaned piglets would improve innate immune defense. An improvement in innate immune defense was defined by increased neutrophil function. It was also hypothesized that supplementation of dietary gln to newly weaned piglets would improve adaptive immune defense. Improved defense is defined as an increase in proliferation in response to mitogens by lymphocytes isolated from blood, mesenteric lymph nodes (MLN) and Peyer's patches (PP) and result from modifications in production of cytokines in peripheral blood and MLN and the proportion of immune cell populations in blood, MLN and PP. It was also hypothesized that isolated intestinal loops challenged with ETEC from gln-supplemented piglets will maintain a) intestinal barrier function and b) functional integrity. Intestinal functional integrity was assessed by measuring potential difference (PD), short-circuit current (Isc) and mannitol flux (ManP).

5.2 MATERIALS AND METHODS

Animals and Diets

Animal studies were reviewed and approved by the Faculty of Agriculture and Forestry Animal Policy and Welfare Committee and were conducted in accordance with the Canadian Council on Animal Care guidelines. Twenty-two female and eighteen male Dutch-Landrace (Genex Swine Group, Heartland Livestock Services, Regina, Saskatchewan) piglets were obtained from the University of Alberta Swine Research and Technology Centre and transported to a separate, temperature-controlled room at the University of Alberta Metabolic Research Unit. Piglets were housed individually in metabolic crates, each fitted with water nipples and creep feeders. Each litter (n=10) was obtained and weaned at approximately 21 days of age. Body weight (BW) and feed intake (FI) were recorded daily during the feeding trial.

Upon receipt of each litter, piglets were assorted randomly into two treatment groups: 1) basal diet with a control mixture of amino acid supplement (ctl) 2) basal diet with gln supplement (gln). These diets were formulated to meet 110% of the requirements for piglet 5-10 kg as specified by the National Research Council (1998) (NRC 1998). A constant portion of the diet is detailed (Table 1) and contained 65 g/kg of fat from a mixture of sources (59.7% Tallow, 33.1% Safflower Oil, and 7.2% Linseed Oil), providing a polyunsaturated to saturated fatty acid ratio of ~ 0.9. The supplement portion of the diet (60 g/kg) consisted of 43.8 g gln for the Gln diet, and the ctl diet consisted of an isomolar, isonitrogenous mixture of amino acids (21.0 g/kg serine, 17.8 g/kg alanine, and 15.0g/kg glycine), which have limited or no metabolic interaction with gln, and are not known to be limiting for growth or immune function in piglets of this age. The variable portion of the diet was made isonitrogenous and isoenergetic by balancing the supplement with sucrose (16.2 g/kg gln diet; 6.2 g/kg ctl).

Sample Collection

At approximately 35 days of age, four animals from each litter were used to study immune function and gut development. Surgical Procedures and sample collection were performed as previously described (Chapter 3). A single blood sample was obtained from each piglet and all subsequent samples were obtained immediately following administration of anaesthetic and surgery.

Immune Measures

As described previously and under sterile conditions, peripheral blood lymphocytes (PBL) and immune cells from MLN (Chapter 3), as well as from PP (Chapter 4) were isolated. The mitogen response assay was conducted in triplicate on microtiter plates as previously described (Chapter 3). Additionally, production of cytokines from immune cells from blood and MLN in complete culture media (CCM; 1×10^6 cells/ml) that were either stimulated with PHA (25 *u*g/ml) or incubated in the absence of mitogen was determined as previously described (Chapter 4). Lymphocyte subsets from freshly isolated immune cells in blood, PP and MLN were identified by indirect immunofluorescence assay as previously described (Chapter 3). The profile of antibodies tested is given in Table 3 of the Appendix. Whole blood was used to assess neutrophil function, as previously described (Chapter 3).

In Situ Surgical Model

Remaining animals from the feeding trial (36-38 days of age) underwent the ***in situ*** closed intestinal loop model of ETEC infection, as described previously (Chapter 3) with some modifications. Briefly, prior to each surgery, *E. coli* cultures were prepared fresh from frozen stock. Fresh cultures of K88 ETEC [K88ac – kindly provided by Dr. Marquardt, University of Manitoba, Winnipeg, MB, Canada (See Appendix for growth curve) and K88 wild type (WT) field isolate, kindly provided by Dr. Nick Nation, VPL laboratories, Edmonton, AB, Canada] were prepared by transferring a small sample of ETEC frozen culture to 6 mls of BHI media (Oxoid LTD., Basingstoke, Hampshire, England) and growing for 24 hrs (in shaking incubator at 37°C). This ETEC culture was subsequently subcultured (24 hrs). The final culture was prepared by transfer of 2 mls of subcultured ETEC media to a 200 ml flask with BHI media (Oxoid) and grown in a shaking incubator at 37^0C for approximately 12 hrs. Samples from these flasks were taken and bacteria were diluted in phosphate-buffered saline (PBS; 300 mOsm/L) to a final concentration of approximately 1 X 10^9 CFU/ml. Because of the concerns that frozen sub-cultures may lose a degree of pathogenicity, the presence of K88 fimbriae was confirmed in each culture by performing a latex agglutination test (Vetway Fimbrex K88, Central Veterinary Laboratory, Surrey, UK) immediately prior to departing the microbiology laboratory to begin the surgical procedure. Following an incubation period of approximately 4 hours, euthanasia was induced (cardiac injection of Pentobarbital Euthanyl – 2 ml/4.5 kg BW), gut sections were removed and immersed in ice-cold PBS.

Ussing Chambers

A small section from each intestinal loop was removed, filled with PBS, and transported to the Ussing apparatus in 50 ml Falcon tubes filled with ice-cold PBS where measurements were made as described previously (Chapter 3).

Statistics

All statistical analyses were completed using the SAS statistical package (Version 8.1, SAS Institute, Cary, NC). Results were presented as means ± SEM. For BW and FI, neutrophil assay, proliferation and phenotype, the effect of diet was determined by one-way analysis of variance (ANOVA), with blocking for piglet litter. Kurtosis and Skew were analyzed for proliferation and cytokine data. Values that were not considered were analyzed for proliferation and cytokine data. Values that were not considered normally distributed by skew and kurtosis values were log transformed, and these values are indicated as log transformed in their respective tables. For Ussing chamber measures and fluid recovery, the effect of diet and loop was determined using a mixed model, with loop comparisons performed by lsmeans, with blocking for piglet litter. Repeated measures analysis was also performed on Ussing chamber data and neutrophil assay data.

5.3 RESULTS

Feed Intake and Weight

Despite randomization, there was a small but significant difference in the mean BW of the groups (5749 ± 320 g ctl vs. 6259 ± 258 g gln) prior to initiating the feeding protocol. Despite this difference, the mean FI, average daily gain (ADG) and final BW did not differ between groups (Table 2).

Neutrophil Function

The absolute production of free radicals (neutrophil burst assessed by mean fluorescence) before and after stimulation did not differ between groups (Table 3). However, there was a significantly lower ($P<0.05$) increase from 0 to 5 and 0 to 10 min. in gln-fed animals, but no difference when assessed between 0 and 15 min. Neutrophils from gln-fed piglets were more granular both before and after stimulation with phorbol myristate acetate (PMA) (Table 3). Repeated measures analysis revealed no significant effect of diet on oxidative burst at the time points studied. However, analysis of the increase betweeen time points revealed a significant diet effect as well as a significant diet*time effect ($P<0.05$).

Immunofluorescence

Expression of several antigens on T cells CD2, CD3, CD4, CD8), B cells (B cell, Immunoglobulin (Ig) A, IgM, PP B cell) and monocytes in PBL (Figure 1), MLN (Figure 2), and PP (Figure 3) were measured. However despite this amount of immune cell characterization, the only significant diet-induced differences where a higher proportion of monocytes in peripheral blood of gln-fed piglets compared to the ctl group (Figure 1) and a lower proportion of IgA+ cells in MLN of gln-fed piglets (Figure 3). The major diet-induced phenotype differences were found with expression of CD45RA. Gln-fed piglets had proportionately less antigen naïve cells (total CD45RA+ expression) in PBL (Table 4) and MLN (Table 5). Expression of this maturation marker was also influenced in PP, where gln-fed piglets were shown to have a decreased proportion of CD4+RA- and CD8+RA- T cells (Table 6). Total expression of this maturation marker in each tissue is shown in Figures 4 (PBL), 5 (MLN) and 6 (PP). Despite the changes in T cell expression of CD45RA, expression of the adhesion molecule (CD44) on T cells (PBL) and B cells (blood and MLN) and total number of CD25+ cells (expression of the IL-2 receptor) did not differ between dietary treatments.

Lymphocyte Proliferation

There was no effect of diet on the rate of ^{3}H-thymidine uptake when cells from peripheral blood, PP or MLN were incubated in the absence of mitogen (Table 7). Neither the absolute rate of ^{3}H-thymidine uptake (decays per minute), nor the stimulation index to any of the mitogens significantly differed in PBL (Table 7). However, in MLN there was a significantly higher ($P<0.05$) SI to pokeweed mitogen (PWM) and lipopolysaccharide (LPS) in gln-fed piglets (Figure 7). Also, in PP of gln-fed piglets there was a significantly higher absolute rate of ^{3}H-thymidine uptake in cells incubated in the presence of PHA ($P<0.03$) and a significantly increased stimulation index following stimulation with LPS ($P<0.01$) (Table 7).

Cytokine Production

Stimulation of PBL with PHA influenced the Th1 response in these animals, with a significantly lower production of IFN-γ in gln-fed piglets (Table 8). This finding in the gln group was also reflected in a significantly decreased IFN-γ/IL-4 ratio, indicative of an increased Th2 response (Table 8). Both Th1- and Th2-type cytokines were influenced in MLN. Incubation of MLN cell with PHA resulted in a significantly lower production of IL-4 and IL-2 in gln-fed animals, suggestive of an increased Th1 response (Table 9). Production of IL-10 and TNF-α were not significantly influenced by diet.

Ussing Chambers

Measurements performed in Ussing Chambers are given in Table 10. Comparison of intestinal loops revealed a significantly higher mannitol permeability (ManP) in loops inoculated with ETEC (K88AC & K88WT) compared to the loops that did not receive ETEC for both diet groups ($P<0.0001$, Figure 8). The volume of fluid in loops that were not inoculated with ETEC was negative (-17 ± 2 mls in ctl-fed piglets (n=6), -23 ± 2 mls in gln-fed piglets (n=6)) compared to loops that were inoculated with K88AC ETEC (8 ± 5 mls in ctl-fed piglets, 11 ± 5 mls in gln-fed piglets) and K88WT ETEC (0 ± 3 mls in in ctl-fed piglets, 7 ± 2 mls in gln-fed piglets). Repeated measures analysis of fluid recovery data revealed a significant loop effect in which AC- and WT-inoculated loops were significantly different than loops that were not inoculated with ETEC (P<0.0001).

Diet did not influence mannitol permeability in either the non- or ETEC-inoculated loops (Figure 8). Electrical measures of intestinal loop tissue mounted in USSING chambers revealed a higher short-circuit current (Isc) in K88AC and K88WT-inoculated loops of control-fed piglets compared to gln-fed piglets (Figure 9). There was also a significantly increased potential difference (PD) in the K88WT-inoculated loops of ctl animals. In the ctl-fed animals there was also an increased PD in K88AC-inoculated loops, but this increase did not reach statistical significance (Figure 10). Although the difference did not reach significance, intestinal loops (that were not exposed to ETEC) from the ctl-fed animals tended to have higher PD and Isc measures than those fed gln (Table 10).

5.4 DISCUSSION

Intake and Growth

Generally, little of the gln provided in the diet directly enters the bloodstream because the absorptive cells of the small intestine utilize almost all that is absorbed from the lumen of the gut (reviewed in (Newsholme & Parry-Billings 1990; Wu 1998)). Therefore the route of gln provision is important. While provision of dietary gln (4% w/w) to young piglets did not increase plasma concentrations of this amino acid (Yoo et al. 1997), increasing the amount of gln (0, 5 and 10%) provided by total parenteral nutrition increased plasma gln concentrations linearly (House et al. 1994)). Although plasma gln concentrations were not measured in the current study, it is expected that plasma gln concentrations would not change with dietary supplementation. Furthermore, these animals were healthy and consuming adequate amounts of diets formulated according to NRC requirements (NRC 1998). The small differences in initial BW between diet groups did not result in significant differences in final BW or the rate of ADG or FI.

Innate Immune Function

Neutrophil function was used as an indication of innate immune function in piglets. Previously, gln has been shown to increase neutrophil function in burn (Ogle et al. 1994) and post-operative patients (Furukawa et al. 1997; Furukawa et al. 2000), and healthy humans (Ogle et al. 1994) and rodents (Pithon-Curi et al. 2002a; Pithon-Curi et al. 2002b; Fukatsu et al. 2003; Ikeda et al. 2003). The beneficial effects of gln include an increase in phagocytosis (Furukawa et al. 2000; Ikeda et al. 2003) and an increased oxidative burst product formation (i.e. production of ROI) (Ogle et al. 1994; Ruggeberg et al. 1997; Furukawa et al. 2000; Fukatsu et al. 2003). Although the preferred energy substrate for neutrophils is glucose, it has been suggested that neutrophils are able to utilize gln when glucose is restricted (Vlessis et al. 1995) and rat neutrophils have been shown to utilize gln at a higher rate than glucose (Pithon-Curi et al. 1997). Additionally, feeding casein (the dietary protein source used in the current study) was shown to stimulate human neutrophil function, but for a less prolonged period of time than feeding a carbohydrate- and protein-free cream preparation (Mohantny et al. 2002). In the current study, neutrophils from gln-fed animals had a lower fold increase (0 to 5, 0 to 10 min) in oxidative burst, although the final production of free radicals did not differ between diets. Although this is not consistent with the literature suggesting that gln enhances neutrophil function, these were healthy animals that were not deficient in gln.

Neutrophil granularity was also measured in the current study, which is influenced by both the number of granules and the amount of proteins and anti-microbial enzymes contained within the granules (Faurschou & Borregaard 2003). Stimulation of neutrophils (with PMA) results in the release of granule proteins (Faurschou & Borregaard 2003) and an increase in granularity, which occurred in neutrophils obtained from both groups. However, in gln-fed piglets, neutrophil granularity was significantly greater at all time points. Previously, neutrophil granularity has been shown to decrease in exercised dogs (Moritz et al. 2003), in horses with inflammatory bowel disease (Weiss & Evanson 2003), during bacterial infection and surgical trauma (Fletcher et al. 1990) and in burn patients (Arturson 1985). Since neutrophils were obtained from healthy piglets, it is difficult to make comparisons to these studies conducted during conditions of stress. However, since a lower granular enzyme content is associated with defective chemotaxis and bactericidal killing by neutrophils (Alexander et al. 1978), the finding of significantly increased neutrophil granularity both before and after stimulation with PMA suggests that gln might positively influence neutrophil function. Although the increase in granularity is important and should be investigated in future studies, the inability to demonstrate a higher oxidative burst suggests that feeding gln to young weaned piglets has a limited influence on this measure of innate immune defense, at least in healthy animals.

Peripheral Immune Function

In vitro, gln is used at a very high rate as a major fuel in lymphocytes (Newsholme & Parry-Billings 1990) and has been shown to influence the lymphocyte proliferative response in a variety of species including humans (Parry-Billings et al. 1990; Rohde et al. 1996; Kohler et al. 2000), mice (Kew et al. 1999), rats (Ardawi & Newsholme 1983; Shewchuk et al. 1997; Liu et al. 2001) and pigs (Yoo et al. 1997). In the present study, contrary to our hypothesis we did not demonstrate that feeding gln significantly increased the rate of ^{3}H-thymidine uptake by peripheral immune cells after mitogen stimulation. This observation was further supported by no differences in IL-2 production between groups. However, it must be considered that unlike these previously mentioned studies, immune cells were isolated from healthy growing animals, which were consuming formulated diets containing high quality proteins. Therefore, gln's contribution to proliferation, as reported in previous clinical studies, may have been blunted due to the presence of sufficient dietary protein in all piglets used in the study.

In peripheral blood, the only significant change among the major immune cell populations occurred in the monocyte population, which was higher in gln-fed piglets. This may simply have resulted from small changes in the proportion of other blood immune cell populations, such as total T cells (CD2+), which were slightly decreased in gln-fed animals. Phenotyping revealed that gln-fed piglets had proportionately less immature/antigen naïve cells (total CD45RA+ expression) in peripheral blood. Expression of CD45 isoforms has been studied previously *in vitro* (Stasny et al. 2001;

Braakman et al. 1991), in models of protein-energy malnutrition in weanling mice (Woodward et al. 1995), in human infants (Field et al. 2000b) and in young piglets (Bozic et al. 2002b; Bozic et al. 2002a; Rodriguez-Carreno et al. 2002). A lower proportion of CD45RA+ cells suggests an increase in antigen exposure/maturation in the gln-fed piglets, but this change could not be attributed to either of the major T cell subsets, and likely explains the similar response to T and B cell mitogens by both groups.

Previously, production of IFN-γ by human PBL stimulated with T cell mitogens has been shown to require an exogenous gln supply (anti-CD3 (Horig et al. 1993), PHA (Rohde et al. 1996) and ConA (Yaqoob & Calder 1998)) and a positive feedback relationship between gln and IFN-γ has been shown (Rohde et al. 1996). Furthermore, the Th1 (increased IFN-γ production), but not Th2, cytokine response to live attenuated bacillus Calmette-Guerin in human peripheral mononuclear cells was shown to require optimal concentrations of gln (Chang et al. 1999). The current study demonstrated that the production of IFN-γ by PBL (reflected in IFN-γ/IL-4 ratio) incubated with PHA was significantly lower in gln-fed animals, which suggests a shift towards a Th2-type response in these animals. This decrease in IFN-γ production by freshly isolated cells from gln-fed piglets is inconsistent with previous findings (reported above) performed by incubating cells *in vitro* with gln. Once might conclude that the influence of diet on cytokine production by peripheral blood cells is more physiologically relevant than in vitro studies and likely closer to the 'true' physiological response. While the mechanism for this change in mitogen-induced cytokine production is not known, the decrease in IFN-γ production in gln-fed animals may have been influenced by the immune cell phenotype of these animals, specifically the lower proportion of CD45RA+ cells. Memory CD45RA- cell from swine (Rodriguez-Carreno et al. 2002) were previously reported to have a higher ability to produce IFN-γ when stimulated with PMA (and calcium ionophore). Additionally, human PBL were found to be capable of producing a much broader spectrum of cytokines than naïve CD45RA+ cells (Jung et al. 1993; Kanegane et al. 1996; Hamann et al. 1997; Mascher et al. 1999). Furthermore, the young age of these piglets may have been a contributing factor as human infants have a predominantly Th2 type cytokine response (Bjorksten 1999). Diet can also influence production of this cytokine, demonstrated previously when infants (6 wks old) fed long chain polyunsaturated fatty acids were shown to produce more IFN-γ than infants fed human milk and commercial term formula (Field et al. 2003).

Mesenteric Lymph Nodes

A higher ^{3}H-thymidine incorporation following stimulation with both PWM (T- and B-cell mitogen) and LPS (strictly B cell mitogen) were observed in the cells from gln-fed piglets. These findings may be due to the proportionately less immature cells (total CD45RA) and higher proportion of CD4+CD45RA- cells in the gln fed animals. Antigen mature cells respond better to mitogens (Stasny et al. 2001). These findings suggest that feeding gln to piglets influences the transition from immune naïve CD45RA+ cells to the mature CD45RA- phenotype in MLN. Despite no significant difference in the rate of ^{3}H-thymidine uptake after PHA stimulation, there was a lower production of IL-2 after PHA stimulation by cells from gln-fed piglets. Lower rates of ^{3}H-thymidine usually accompany lower IL-2 production and this observation requires further study.

Stimulation of MLN cells with PHA also resulted in a significantly lower production of IL-4 in gln-fed animals.

Peyer's Patches

Log transformation of PP proliferation data revealed a significant high rate of ^{3}H-thymidine uptake in cells from gln-fed piglets after stimulation with PHA and significantly increased stimulation index following stimulation with LPS. This increased proliferative rate was accompanied by a decrease in the proportion of CD4+RA- and CD8+RA-. While T cells which have previously encountered antigen are thought to be better able to respond to T cell mitogens, it is important to note that several RA isoforms exist in swine (RAC, RA, RC, RO (Haverson et al. 1999)). Therefore it is difficult to conclude that the decrease in RA- T cells in gln-fed piglets was actually a decrease in the proportion of T cells that had been previously exposed to antigen. These results may also have been influenced by the considerable variation in the mitogen response between piglets and that, compared to cells from other tissues, cells from PP responded poorly to mitogens used in the present study. Unfortunately, it was not possible to measure cytokine production by PP cells, which may have clarified these findings. Therefore, a more detailed analysis of the influence of gln on PP lymphocyte function and maturation is warranted.

ETEC Infection Model – Intestinal Permeability

The ETEC model developed in our lab was used to study the initial stages of infection in the small intestine, examining the effect *in vitro* by mounting tissue in Ussing chambers.

Measuring unidirectional serosal-to-mucosal flux of mannitol, thus changes in the flux of this hydrophilic solute, reflects changes in the permeability of the paracellular pathway. A significantly higher ManP was found in loops inoculated with ETEC (K88AC & K88WT), indicating that the presence of ETEC in loops increases intestinal permeability. No significant effect of diet on ManP was found. However, since an increase in surface area would also increase ManP, potential diet-induced changes in surface area would need to be excluded before concluding with complete confidence that diet has no effect on this measure. An increase in permeability due to the presence of pathogens has been reported previously in studies of *in vivo* infection by transmissible gastroenteritis virus (Keljo et al. 1985) and rotavirus (Isolauri et al. 1993) in piglets and rats, respectively. The increased paracellular permeability observed in the above studies was suggested to be caused by either the immune response to these bacteria or the actions of the bacteria themselves. Although we predicted that gln would maintain intestinal barrier function, permeability in ETEC-inoculated loops did not differ between ctl- and gln-fed piglets.

ETEC Infection Model – Intracellular Ion Movement

ETEC produce enterotoxins (heat-stable & heat-labile) following adherence to an intestinal epithelial cell (reviewed in (Gyles 1992)) which cause an increase in intracellular cGMP and cAMP, respectively. Overproduction of these cyclic nucleotides both inhibits the absorption of electrolytes by the cells of the villus and stimulates a sustained increase in Cl- secretion by the cells of the crypt through phosphorylation of specific Cl- channels. This rapid increase in the rate of secretion of electrolytes and water from the intestine leads to diarrhea. This was confirmed in the current study, as shown by the increased fluid absorption in non-ETEC exposed loops (demonstrated by the decreased recovery of liquid from these loops). While jejunal intestinal sections from this study did not demonstrate a significant change in Conductance (G) or the Forskolin (Fors) response (change in Isc) with ETEC exposure, ctl-fed piglets demonstrated significantly higher Isc (K88AC & K88WT loops) and PD (K88WT loop) electrical measures compared to gln-fed piglets after ETEC exposure. Tissue Isc in Ussing chambers is a measure of the net movement of several actively transported ions, which significantly increases following addition of heat-stable enterotoxin to rabbit distal ileal sections (Guandalini et al. 1982). PD is also related to ion movement, only it measures changes in the electrochemical gradient across the intestinal epithelium that is generated by electrogenic ion pumps in epithelial cell membranes, mainly the Na-K-ATPase (Armstrong et al. 1987). Therefore, there are two possible explanations for the higher values in ctl-fed piglets. Since ion movement was increased, the enterocytes from these loops were either absorbing or secreting ions to a greater extent than enterocytes in gln-fed piglets. Since movement of individual ions was not determined in this study, it is difficult to determine with absolute certainty whether secretion and/or absorption was influenced. Previous studies have shown that gln stimulates electrogenic Na+ absorption and electrically silent NaCl absorption (cultured Caco-2 cells (Rhoads et al. 1990; Rhoads et al. 1992)) and stimulates Na+ and NaCl transport in rotavirus-damaged jejunum of piglets (6-20 days of age) (Rhoads et al. 1991). Based on these previous findings, it is less likely that ctl-fed animals had an increased rate of ion absorption. The more likely explanation for the increased Isc and PD in ctl-fed animals was an increased Cl- secretion caused by ETEC enterotoxins. It is possible that enterocytes of gln-fed piglets were not influenced to the same extent by the presence of ETEC, either by being less responsive to secreted enterotoxins or because ETEC was not able to bind and release enterotoxins to the same magnitude. The appearance of the intestinal tissues of the gln-fed piglets was different from that of the control tissue. This suggest that the expression of enterocyte surface molecules (i.e. glycoprotein and/or glycolipids) may have been influenced by feeding gln, but this requires further research. Additionally, since the current study demonstrated that feeding gln also influences immune cells populations and function, these changes may have influenced gut function and future studies are warranted to determine the interactions which occur in this tissue.

Summary and Conclusion

It was hypothesized that supplementation of dietary gln to newly weaned piglets would improve innate immune defense. Although the increase in neutrophil granularity suggests an improvement in innate immune defense, the inability to demonstrate a higher oxidative burst suggests that feeding gln to young weaned piglets has a limited influence on this measure of peripheral immune defense. A more detailed analysis of gln as an energy source and whether gln influences expression of intracellular protein are warranted in future studies. It was also hypothesized that supplementation of dietary gln to newly weaned piglets will improve adaptive immune defense. The influence of gln on peripheral immune function was limited to decreasing the proportion of antigen naïve cells and decreasing the production of IFN-γ after stimulation. However, these unique *in vivo* findings may be related and require further study. An improvement in adaptive immune defense by GALT immune cells was suggested by the higher incorporation of

^{3}H-thymidine in MLN (to PWM and LPS) and PP lymphocytes (to PHA and LPS). This improvement was further demonstrated in MLN by the lower proportion of immature cells in gln-fed piglets. It must also be considered that the majority of advocate studies for the benefits of gln have been conducted in patients/animals during times of considerable "stress". The healthy condition of the animals studied and the glutamine content of the basal diet may have blunted the effect of supplemental gln. Also, the ability of gln to influence any function beyond the catabolic cells of the intestinal mucosa has been debated. However, the interaction of dietary gln with GALT lymphocytes produced several interesting findings and studies are warranted to study GALT immune cells function *in vivo*. It was also hypothesized that isolated intestinal loops challenged with ETEC from gln-supplemented piglets will maintain intestinal barrier function and functional integrity. Despite clear changes in permeability in ETEC-inoculated loops, there was not an observed diet-induced difference in permeability. The significantly lower PD and Isc suggests that feeding gln may have influenced enterocyte surface molecules, which in turn may have influenced the ability of ETEC to bind and bring about increases in ion secretion. A more detailed analysis of expression of intracellular mediators and tight junction proteins following ETEC exposure is warranted in future studies. In conclusion, providing 4.4% w/w gln to nutritionally adequate weaning diets influences immune and gut function and future studies are warranted to determine the mechanisms and interrelationships.

TABLE 1. Composition of experimental diets

		Diet (g/kg)	
Ingredient		**Ctl**	**Gln**
WHEAT		382	382
SOYBEAN MEAL		232	232
CASEIN		232	232
FAT - Tallow		38.8	38.8
- Safflower Oil		21.5	21.5
- Linseed Oil		4.70	4.70
LIMESTONE		6.00	6.00
DICALCIUM PHOSPHATE		18.9	18.9
SALT		2.00	2.00
SWINE VITAMIN/MINERAL PREMIX[1]		0.750	0.750
PC Swine Trace Mineral 3[2]		1.00	1.00
Vitamin B12		1.67×10^{-5}	1.67×10^{-5}
SUPPLEMENT - Gln			43.8
- Serine		21.0	
- Alanine		17.8	
- Glycine		15.0	
- Sucrose		6.20	16.2

[1] The vitamin premix contained: 3.8912% Protein, 0.99% Fat, 2.701% Moisture, 86.002 kcal/kg DE, 82.0002 kcal/kg ME, 25.7937% Calcium, 0.225% Phosphorus, 0.0765% Av Phosphorus, 0.1282% Magnesium, 0.0328% Sodium, 642.2313 mg/kg Iron, 7000014.2 IU/kg Vitamin A, 700014.2 IU/kg Vitamin D3, 20000.0117 IU/kg Vitamin E, 1500.0179 mg/kg Vitamin K, 40.0001 mg/kg Biotin, 399.9008 mg/kg Folic Acid, 20000.0117 mg/kg Niacin, 7499.9741 mg/kg Pantothenic Acid, 533.9845 mg/kg Pyridoxine, 3000.0051 mg/kg Riboflavin, 580.5812 mg/kg Thiamine, 10 mg/kg Vitamin B12

[2] The trace mineral premix contained: 0.99% fat, 0.001% moisture, 86 kcal/kg DE, 82 kcal/kg ME, 15.1874% Calcium, 0.0729% Magnesium, 0.0283% Sodium, 4.756% Sulfur, 351 mg/kg Cobalt, 5000 mg/kg Copper, 749.3 mg/kg Iodine, 75365.1328 mg/kg Iron, 25020 mg/kg Manganese, 150 mg/kg Selenium, 75024 mg/kg Zinc, 100.001 g/kg Choline

TABLE 2. Body weight and feed intake of piglets fed gln and ctl diets

Dietary Group	**Ctl (n=20)**	**Gln (n=20)**
Initial BW, *g*	5749^{a}[1,2] ± 320	6259^{b} ± 258
Final BW, *g*	7740 ± 422	8155 ± 423
ADG, g	1990 ± 199	1896 ± 248
FI, *g/d*	174 ± 16	172 ± 16
FI, *g/kg BW*	24.67 ± 2	22.22 ± 2

[1] Values presented as mean ± SEM

[2] Means within a row that do not share a common letter are significantly different ($p<0.05$)

TABLE 3. Neutrophil Burst, Size and Granularity measures of piglets supplemented with Gln or Ctl diets (35 days of age)

Measure	Time	Ctl (n=10)	Gln (n=10)
Burst	0 min.	6[1] ± 1	8 ± 1
	5 min.	87 ± 13	72 ± 10
	Fold increase (0-5 min.)[2]	19[3]^{b} ± 3	12^{a} ± 3
	10 min.	358 ± 55	279 ± 34
	Fold increase (0-10 min.)	75^{b} ± 11	44^{a} ± 9
	Fold increase (5-10 min.)	4 ± 0.2	4 ± 0.2
	15 min.	773 ± 103	674 ± 78
	Fold increase (0-15 min.)	168 ± 30	109 ± 22
	Fold increase (10-15 min.)	2 ± 0.2	2 ± 0.1
Size	0 min.	503 ± 17	516 ± 17
	5 min.	531 ± 19	539 ± 20
	10 min.	565 ± 17	568 ± 18
	15 min.	569 ± 13	575 ± 14
Granularity	0 min.	280^{a} ± 7	300^{b} ± 10
	5 min.	291^{a} ± 5	311^{b} ± 8
	10 min.	292^{a} ± 4	312^{b} ± 8
	15 min.	300^{a} ± 5	340^{b} ± 16

1 Values presented as mean fluorescence ± SEM

[2] Repeated measures analysis of the neutrophil oxidative burst fold increase revealed a significant effect at each time point, as well as a significant diet*time effect ($p<0.05$)

[3] Means within a row that do not share a common letter are significantly different ($p<0.05$)

Table 4. Cell Phenotyping from PBL of piglets fed Gln and Ctl diets

Antibody	Ctl (n=10)	Gln (n=10)
IGA	9[1] ± 1	7 ± 1
IGM	16 ± 1	16 ± 2
CD3+CD4+	41 ± 5	41 ± 7
CD3+CD8+	23 ± 5	25 ± 5
CD44+BCell+	18 ± 3	18 ± 3
% B cell CD44+	90 ± 3	95 ± 1
CD44+CD3+	44 ± 13	47 ± 12
% CD3 CD44+	97 ± 1	98 ± 1
CD4+CD45RA+	29 ± 5	25 ± 4
CD4+CD45RA-	12 ± 2	13 ± 3
CD8+CD45RA+	23 ± 2	20 ± 2
CD8+CD45RA-	5 ± 1	5 ± 1
CD2+CD3+	28 ± 5	24 ± 5
CD2+CD3-[3]	14 ± 3	16 ± 3
CD3+	54 ± 4	52 ± 4
CD4+	50 ± 4	43 ± 3
CD8+	29 ± 3	28 ± 4
CD4/CD8	2 ± 0.3	2 ± 0.3
CD25+	24 ± 2	25 ± 2
Ra+	66^{b}[2] ± 3	61^{a} ± 4
CD44+	95 ± 1	96 ± 1
Monocyte+	16^{a} ± 1	19^{b} ± 1
B cell+	24 ± 2	25 ± 3
CD2+	54 ± 9	50 ± 7

[1] Values presented as cell population percentage ± SEM

[2] Means within a row that do not share a common letter are significantly different ($p<0.05$)

[3] This population was assumed to be the CD4CD8 double negative T cell subset

Table 5. Cell Phenotyping from MLN Lymphocytes from piglets fed Gln and Ctl diets

Antibody	Ctl (n=10)	Gln (n=10)
IGA	17^{b}[2] ± 1	10^{a} ± 1
IGM	22 ± 2	18 ± 3
CD4+CD45RA+	41 ± 4	33 ± 3
CD4+CD45RA-	13^{a} ± 2	19^{b} ± 3
CD8+CD45RA+	30 ± 2	29 ± 2
CD8+CD45RA-	6 ± 1	7 ± 1
CD4+CD25+	15 ± 2	13 ± 2
CD8+CD25+	16 ± 3	17 ± 3
CD2+CD3+	33 ± 3	36 ± 5
CD2+CD3-[3]	28 ± 6	23 ± 4
CD3+	51 ± 2	51 ± 4
CD4+	49 ± 2	50 ± 3
CD8+	38 ± 3	37 ± 3
CD4/CD8	1 ± 0.1	1 ± 0.2
CD25+	22 ± 3	21 ± 3
Ra+	77^{b} ± 2	69^{a} ± 4
B cell+	39 ± 4	36 ± 4
CD2+	61 ± 8	59 ± 8

[1] Values presented as cell population percentage ± SEM

[2] Means within a row that do not share a common letter are significantly different ($p<0.05$)

[3] This population was assumed to be the CD4CD8 double negative T cell subset

TABLE 6. Cell Phenotyping from PP of piglets fed Gln and Ctl diets

Antibody	**Ctl (n=10)**	**Gln (n=10)**
IGA	5 ± 1	4 ± 1
IGM	40 ± 3	42 ± 3
PP BCELL	20 ± 4	13 ± 3
CD44+BCELL+	9 ± 2	11 ± 3
% Bcell CD44+	27 ± 7	29 ± 7
CD4+CD45RA+	17 ± 7	9 ± 2
CD4+CD45RA-	5^{b}[2] ± 1	3^{a} ± 1
CD8+CD45RA+	14 ± 3	12 ± 3
CD8+CD45RA-	7^{b} ± 3	4^{a} ± 1
CD3+	25 ± 3	20 ± 3
CD4+	22 ± 7	12 ± 2
CD8+	21 ± 6	15 ± 4
CD4/CD8	1.5 ± 0.6	1.0 ± 0.3
RA+	82 ± 4	86 ± 3
CD44+	31 ± 6	29 ± 6
Monocyte+	21 ± 3	19 ± 4
B cell+	46 ± 4	46 ± 3
CD2+	33 ± 3	25 ± 3

[1] Values presented as cell population percentage ± SEM

[2] Means within a row that do not share a common letter are significantly different ($p<0.05$)

TABLE 7. ^{3}H-Thymidine Uptake by PBL, MLNL, and PPL in piglets fed Gln and Ctl diets and taken at 35 days of Age

Tissue (Mitogen)		Ctl (n=10)	Gln (n=10)
PBL (none)	**DPM**	1036[1] ± 429	2303 ± 850
(PHA)	**DPM**	287271 ± 47681	303822 ± 43258
Log transformed[2]	**SI**	2.6[3] ± 0.1	2.5 ± 0.2
(PWM)	**DPM**	162828 ± 28855	126579 ± 25614
Log transformed	**SI**	2.3 ± 0.2	2.0 ± 0.2
(LPS)	**DPM**	2900 ± 907	3306 ± 920
Log transformed	**SI**	0.21 ± 0.4	-0.09 ± 0.4
MLN (none)	**DPM**	236 ± 30	173 ± 25
(PHA)	**DPM**	291989 ± 41485	309169 ± 42779
	SI	1745 ± 457	2300 ± 456
(PWM)	**DPM**	203420 ± 28835	233628 ± 30501
	SI	1130[a][4] ± 254	1675[b] ± 297
(LPS) Log transformed	**DPM**	2.5 ± 0.1	2.8 ± 0.2
Log transformed	**SI**	-0.13[a] ± 0.07	0.32[b] ± 0.2
PP (none)	**DPM**	67 ± 11	76 ± 15
(PHA) Log transformed	**DPM**	2.7[a] ± 0.1	3.3[b] ± 0.2
Log transformed	**SI**	0.8 ± 0.09	1.4 ± 0.2
(PWM) Log transformed	**DPM**	2.3 ± 0.1	2.6 ± 0.2
	SI	3 ± 1	5 ± 1
(LPS) Log transformed	**DPM**	1.8 ± 0.1	2.0 ± 0.2
Log transformed	**SI**	-1.0[a] ± 0.3	-0.2[b] ± 0.3

[1] Values reported in mean Decays per Minute± SEM

[2] Kurtosis and Skew were analyzed for proliferation data and values that were not considered normally distributed were log transformed.

[3] Values presented as mean Stimulation Index ± SEM, calculated as DPM stimulated/DPM unstimulated

[4] Means within a row that do not share a common letter are significantly different ($p<0.05$)

TABLE 8. Cytokine Production by PHA in PBL in piglets fed Gln and Ctl diets and taken at 35 days of Age

Cytokine	Ctl (n=10)	Gln (n=10)
IL-4 (log transformed)[1]	1.7[2] ± 0.1	1.6 ± 0.06
IL-2 (log transformed)	2.4 ± 0.2	2.1 ± 0.2
IFN-γ (log transformed)	2.3[b][3] ± 0.2	1.9[a] ± 0.1
TNF-α	32 ± 3	33 ± 5
IL-10 (log transformed)	1.1 ± 0.2	1.0 ± 0.1
IFN-γ/IL-4 (log transformed)	0.59[b] ± 0.1	0.23[a] ± 0.07

[1] Kurtosis and skew were analyzed for cytokine data and values that were not considered normally distributed were log transformed.

[2] Values presented as mean concentration ± SEM

[3] Means within a row that do not share a common letter are significantly different ($p<0.05$)

TABLE 9. Cytokine Production by PHA in Mesenteric Lymph Node Lymphocytes in piglets fed Gln and Ctl diets and taken at 35 days of Age

Cytokine	Ctl (n=10)	Gln (n=10)
IL-4	259[b][1,2] ± 36	146[a] ± 19
IL-2 (log transformed)[3]	2.9[b] ± 0.1	2.4[a] ± 0.2
IFN-γ (log transformed)	2.4 ± 0.1	2.5 ± 0.2
TNF-α	32 ± 2	35 ± 3
IL-10	58 ± 12	74 ± 14
IFN-γ/IL-4 (log transformed)	0.05 ± 0.1	0.4 ± 0.2

[1] Values presented as mean concentration ± SEM

[2] Means within a row that do not share a common letter are significantly different ($p<0.05$)

[3] Kurtosis and skew were analyzed for cytokine data and values that were not considered normally distributed were log transformed.

Table 10. Permeability and Electrical Measures of intestinal loops in USSING chambers from piglets supplemented with Gln or Ctl diets (35 days of age)

Measure	Ctl (n=10)	Gln (n=10)
ManP (nm/cm/hr)		
Non-ETEC	$13.4^{y1} \pm 1.28$	$16.1^{y} \pm 2.46$
K88 AC Loop	$22.7^{z} \pm 2.65$	$25.1^{z} \pm 1.87$
K88 WT Loop	$22.2^{z} \pm 3.85$	$22.3^{z} \pm 1.96$
G (mS/cm^2)		
Non-ETEC	19.4 ± 2.70	19.7 ± 2.00
K88 AC Loop	19.1 ± 2.09	20.5 ± 2.91
K88 WT Loop	17.2 ± 1.29	19.9 ± 1.44
Isc (uA/cm2)		
Non-ETEC	17.6 ± 2.93	10.3 ± 1.57
K88 AC Loop	$24.2^{b} \pm 3.88$	$12.7^{a} \pm 2.05$
K88 WT Loop	$22.4^{b} \pm 2.24$	$13.0^{a} \pm 3.07$
PD (mV)		
Non-ETEC	1.00 ± 0.190	0.548 ± 0.100
K88 AC Loop	1.34 ± 0.306	0.789 ± 0.262
K88 WT Loop	$1.36^{b} \pm 0.227$	$0.628^{a} \pm 0.151$
Fors (Δ Isc)		
Non-ETEC	41.9 ± 3.69	41.0 ± 5.07
K88 AC Loop	53.0 ± 5.29	47.6 ± 6.45
K88 WT Loop	41.9 ± 8.92	39.3 ± 4.57

[1] Values presented as mean ± SEM

[2] Diet, Loop and Block statistical analysis performed by SAS mixed model where loop comparisons performed by lsmeans with significant comparisons at the 0.05 level are indicated with different letters. Repeated measures analysis revealed a significant loop effect (P<0.0001) for Mannitol, and significant diet effects for PD and Isc (P<0.0001)

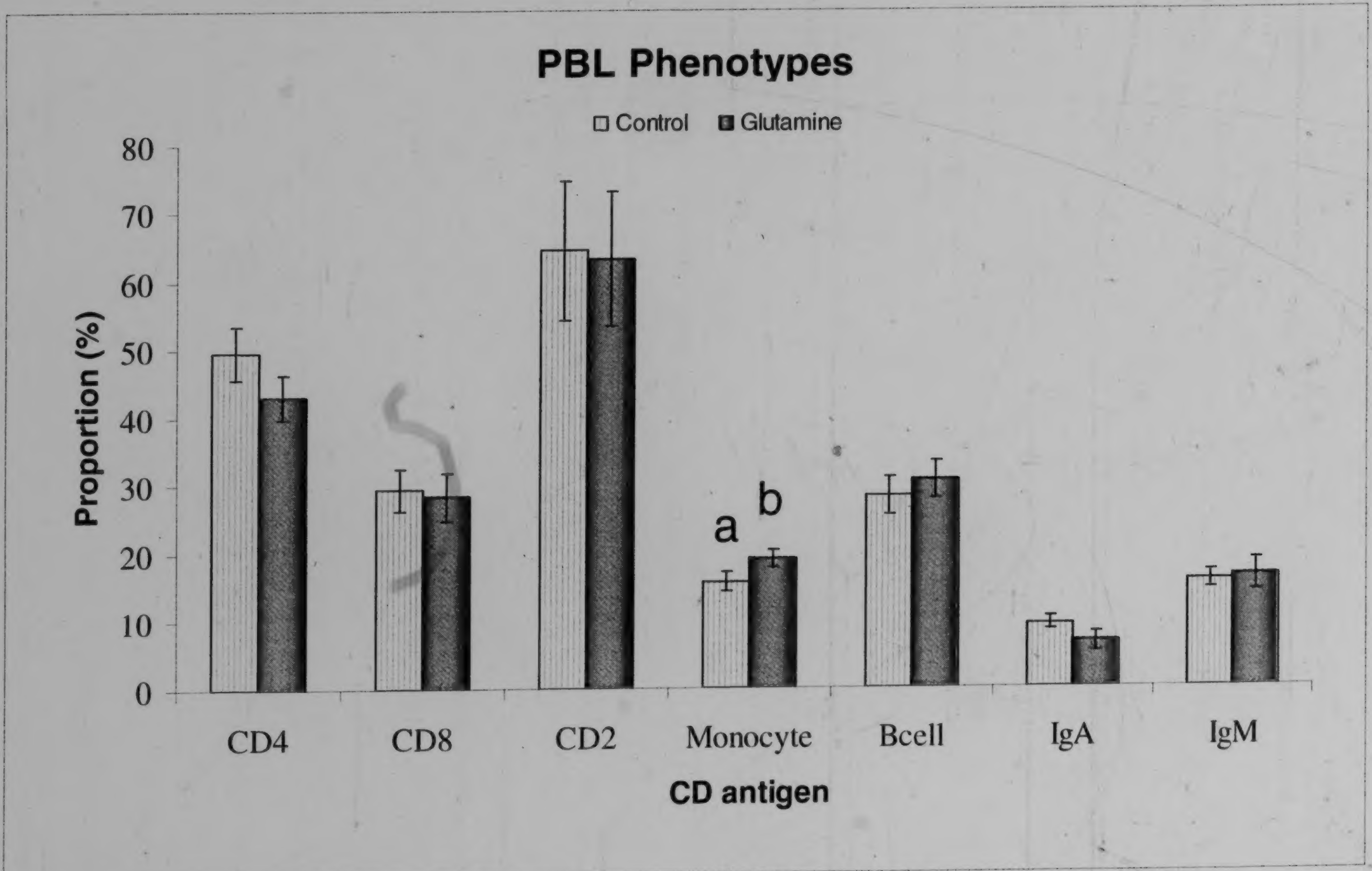


Figure1. Influence of diet on PBL Phenotypes. Phenotypes given as mean with error bars representing SEM. Phenotypes that do not share a common letter are significantly different ($p<0.05$).

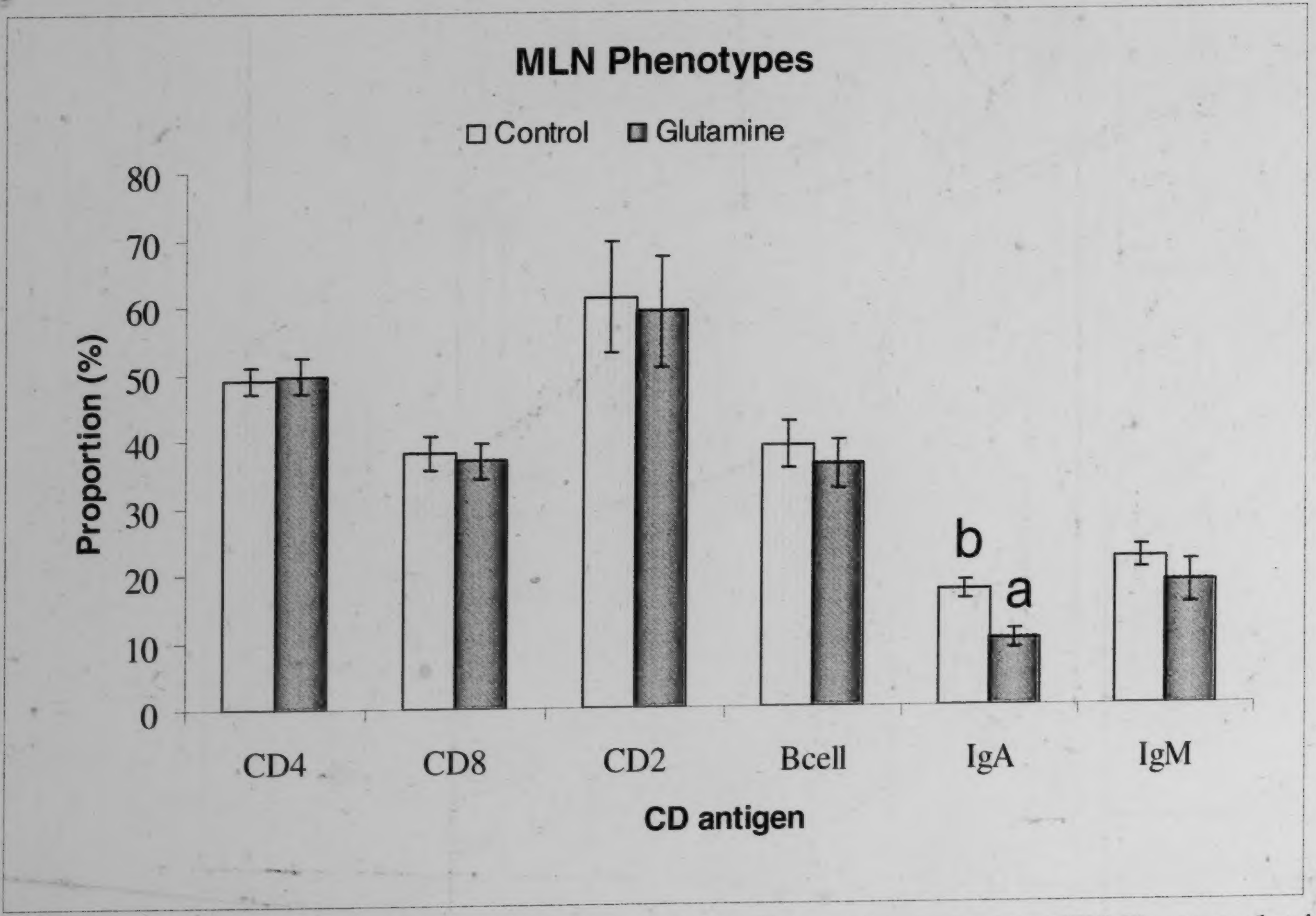


Figure 2. Influence of diet on MLN Phenotypes. Phenotypes given as mean with error bars representing SEM. Phenotypes that do not share a common letter are significantly different (p<0.05).

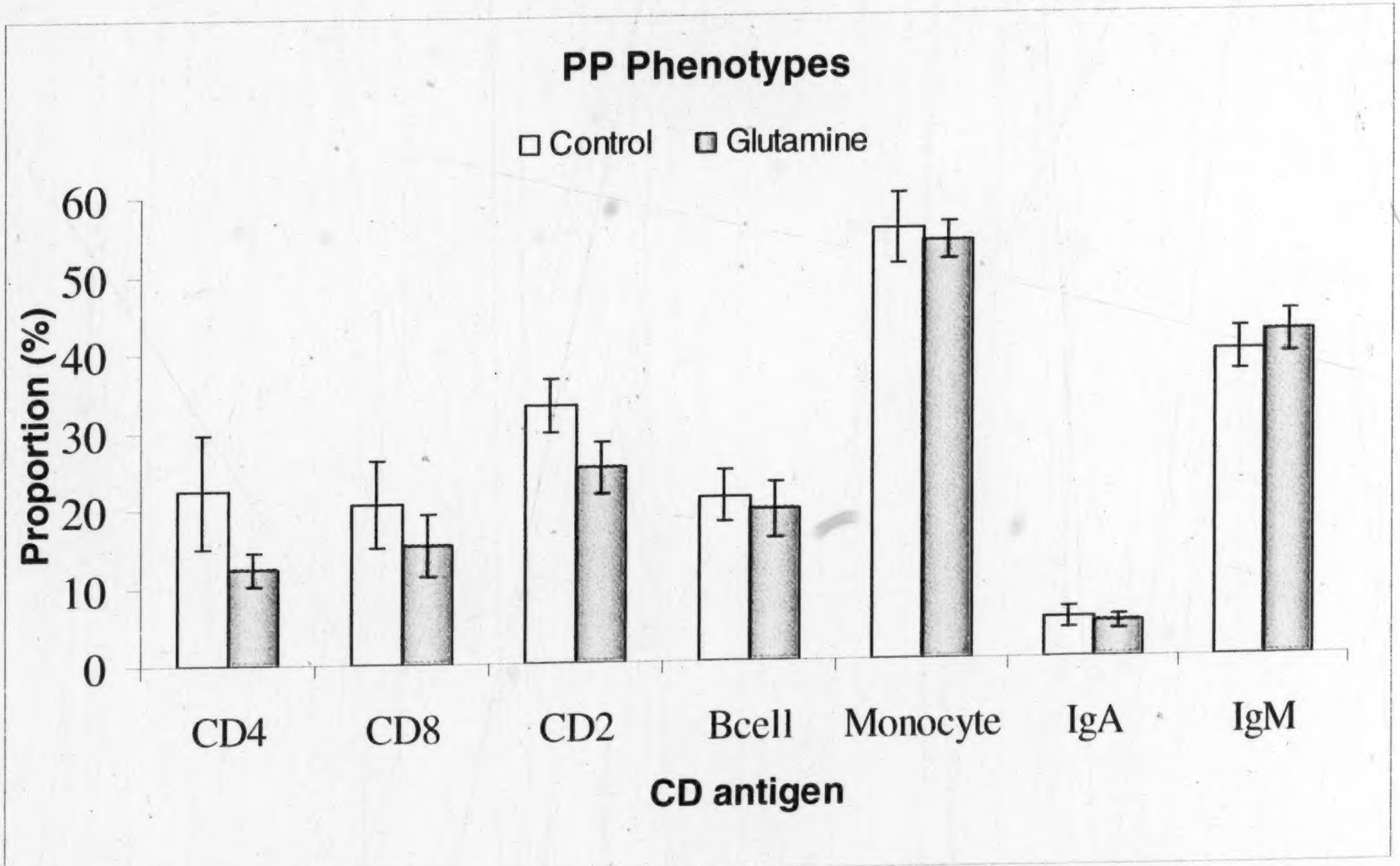


Figure 3. Influence of diet on PP Phenotypes. Phenotypes given as mean with error bars representing SEM. PP Phenotypes were not significantly different ($p<0.05$).

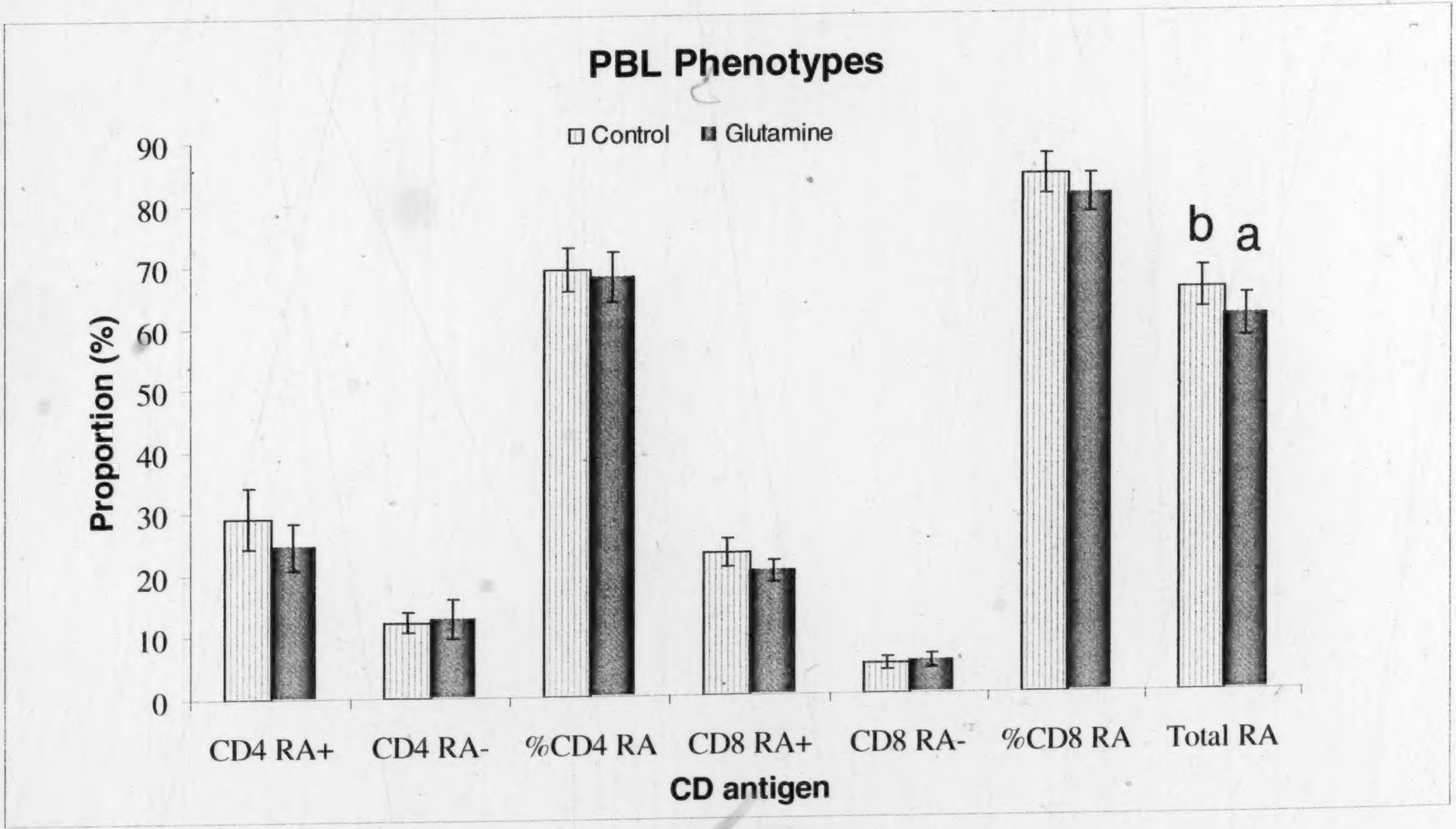


Figure 4. Influence of diet on PBL expression of CD45RA. Phenotypes given as mean with error bars representing SEM. Phenotypes that do not share a common letter are significantly different ($p<0.05$).

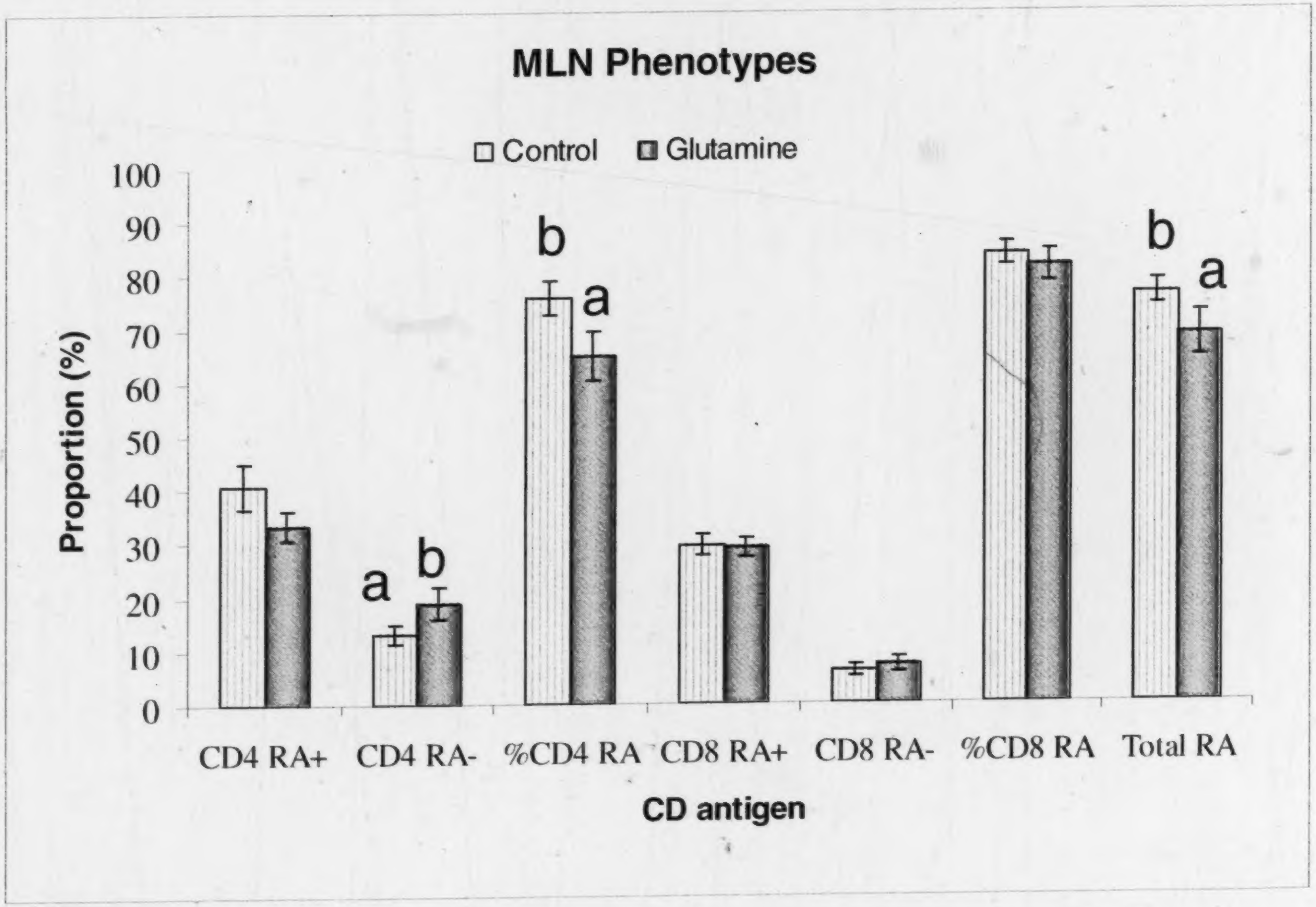


Figure 5. Influence of diet on MLN expression of CD45RA. Phenotypes given as mean with error bars representing SEM. Phenotypes that do not share a common letter are significantly different ($p<0.05$).

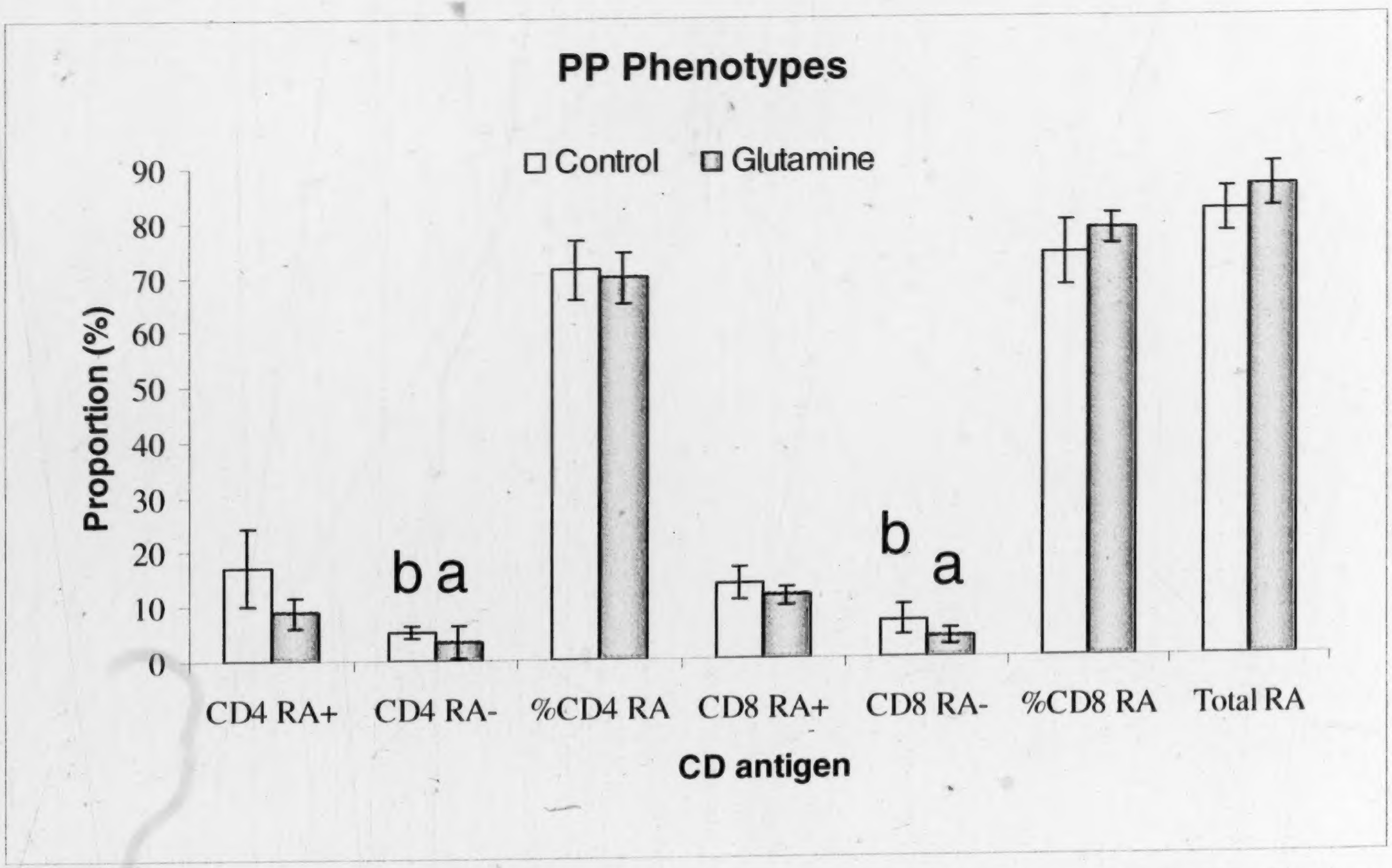


Figure 6. Influence of diet on PP expression of CD45RA. Phenotypes given as mean with error bars representing SEM. Phenotypes that do not share a common letter are significantly different ($p<0.05$).

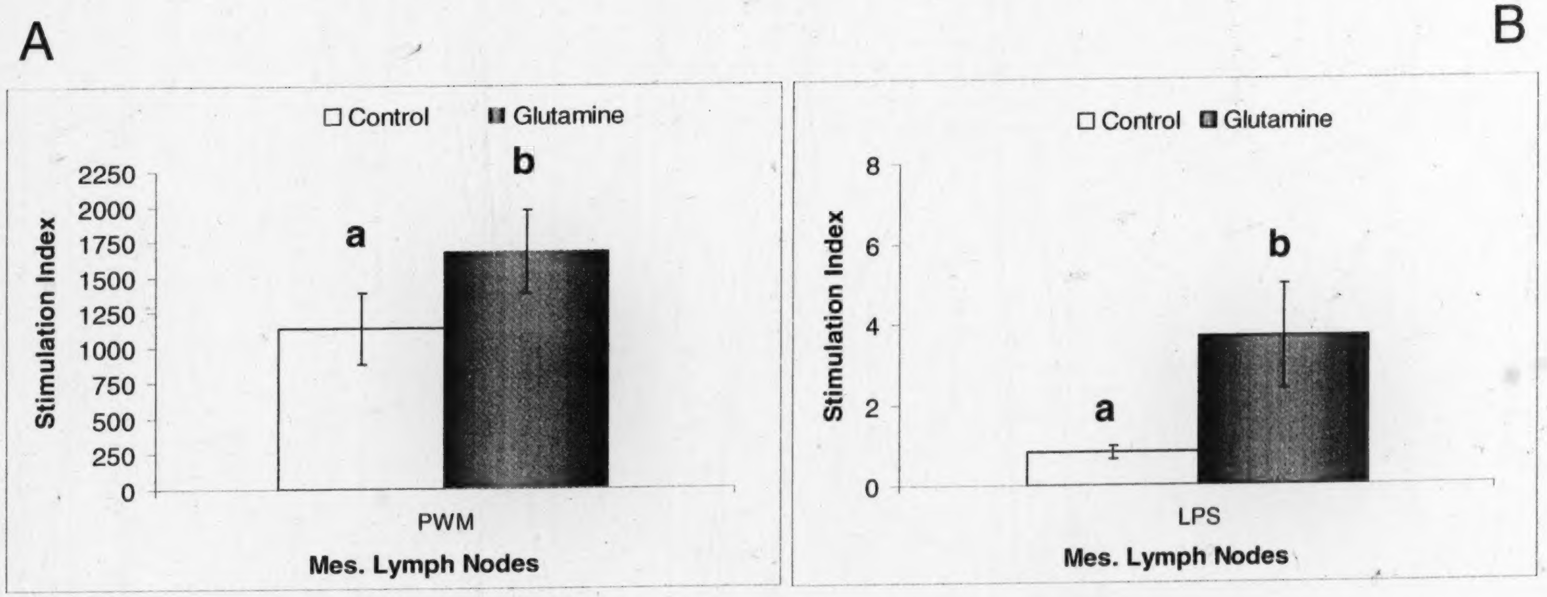


Figure 7. Lymphocyte proliferation stimulation indices of MLN lymphocytes isolated from piglets fed gln or ctl diets. Lymphocytes were stimulated with PWM (A) and LPS (B). Indicated as mean stimulation index (SI) with error bars representing SEM. SIs that do not share a common letter are significantly different ($p<0.05$).

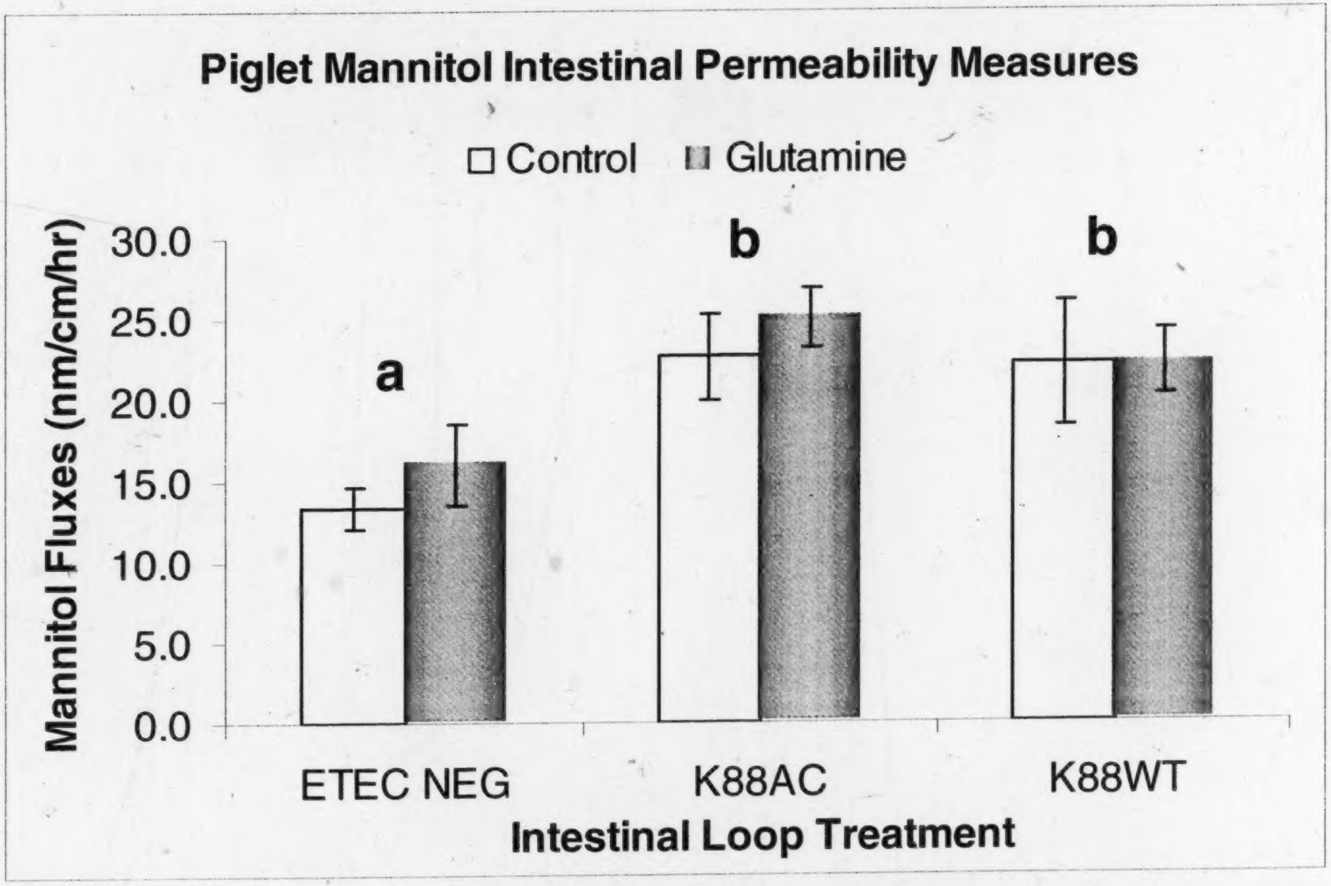


Figure 8. Jejunal ManP of tissues isolated from piglets fed gln and ctl diets that underwent the *in situ* surgical procedure. Indicated as mean mannitol flux (nm/cm/hr) with error bars representing SEM. Loops that do not share a common letter are significantly different ($p<0.05$).

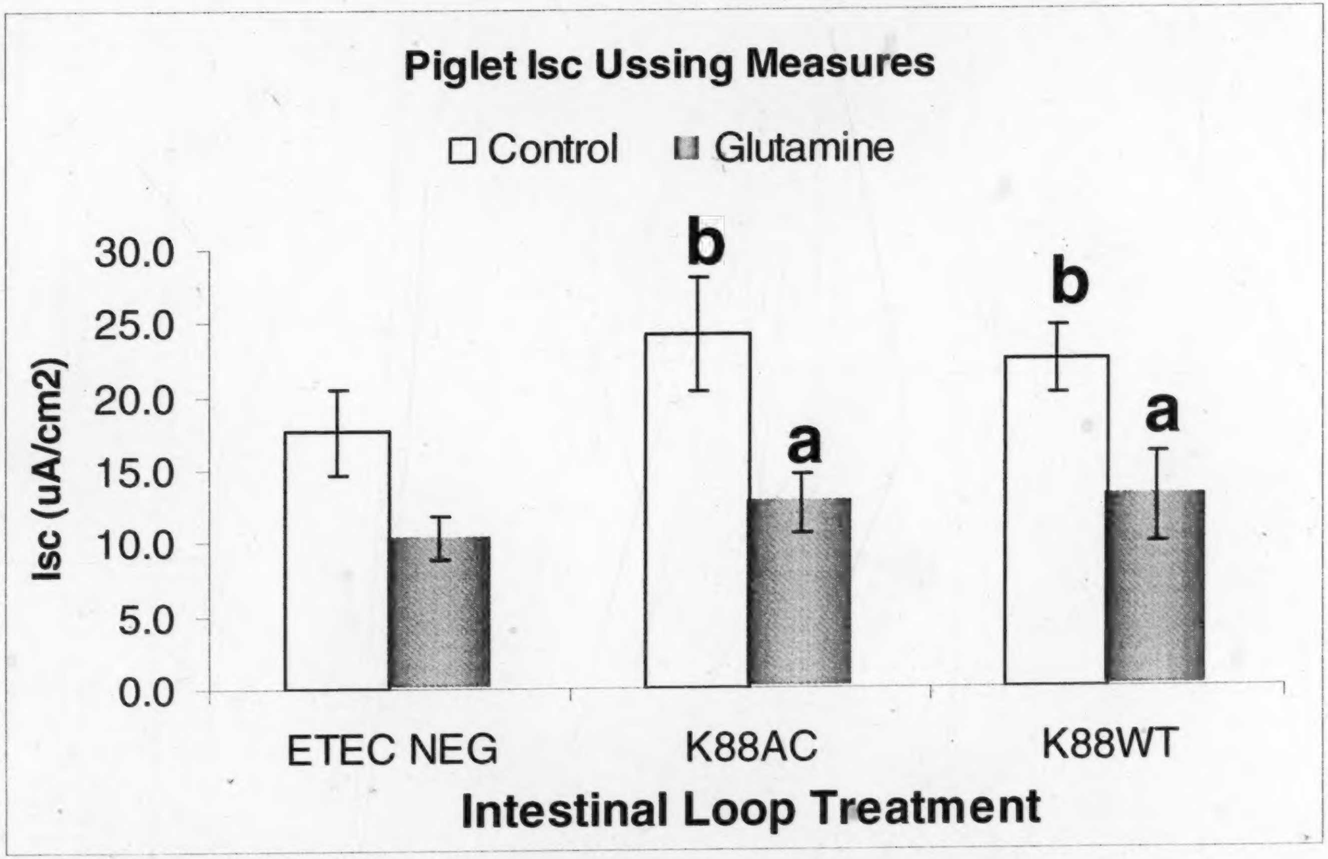


Figure 9. Jejunal Isc of tissues isolated from piglets fed gln and ctl diets that underwent the *in situ* surgical procedure. Indicated as mean Isc (uA/cm^2) with error bars representing SEM. Diets that that do not share a common letter are significantly different ($p<0.05$).

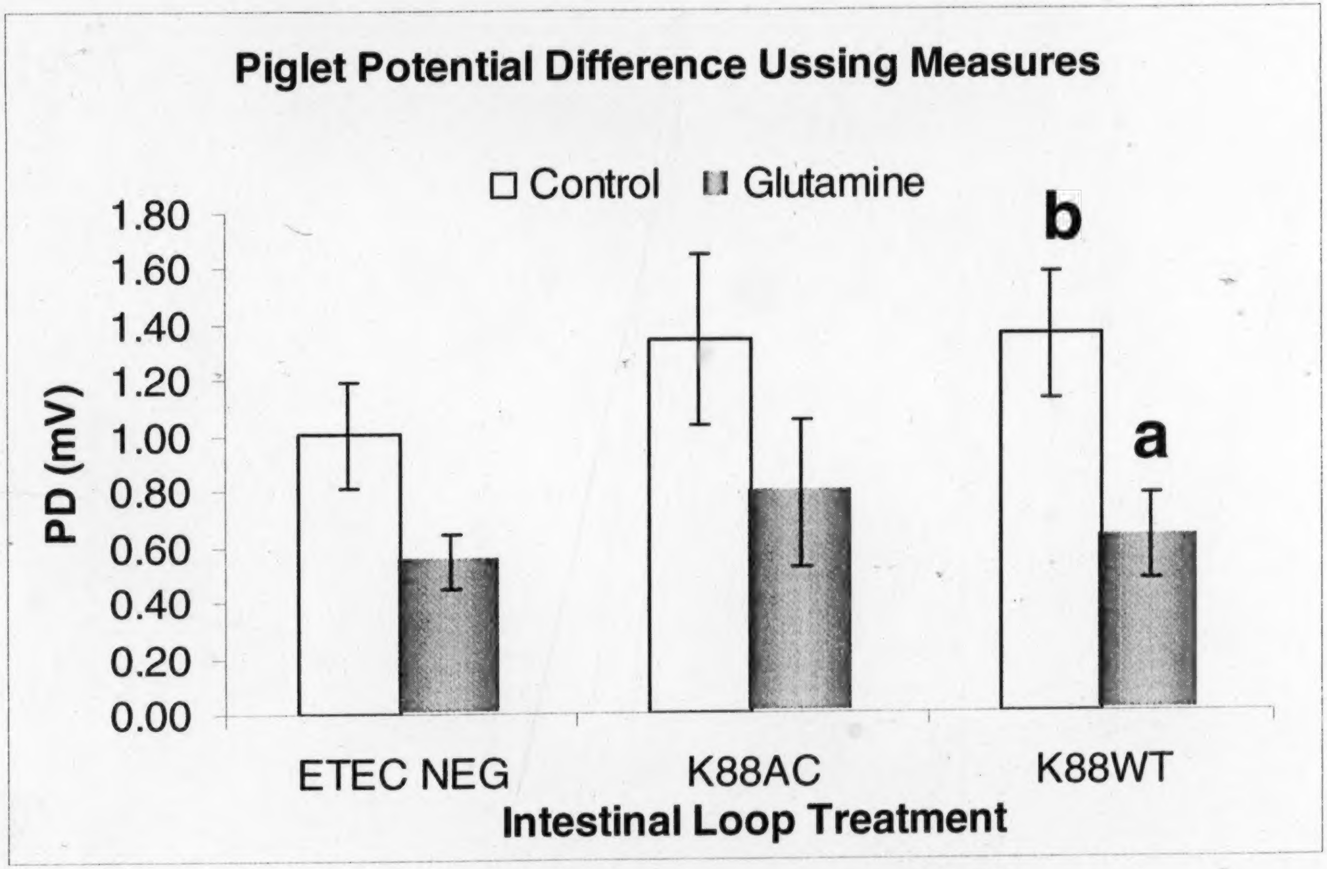


Figure 10. Jejunal PD of tissues isolated from piglets fed gln and ctl diets that underwent the *in situ* surgical procedure. Indicated as mean PD (mV) with error bars representing SEM. Diets that that do not share a common letter are significantly different ($p<0.05$).

5.5 REFERENCES

Adegoke,O.A., McBurney,M.I., and Baracos,V.E. 1999a. Jejunal mucosal protein synthesis: validation of luminal flooding dose method and effect of luminal osmolarity. Am. J. Physiol 276: G14-G20.

Adegoke,O.A., McBurney,M.I., Samuels,S.E., and Baracos,V.E. 1999b. Luminal amino acids acutely decrease intestinal mucosal protein synthesis and protease mRNA in piglets. J. Nutr. 129: 1871-1878.

Alexander,J.W., Ogle,C.K., Stinnett,J.D., and Macmillan,B.G. 1978. A sequential, prospective analysis of immunologic abnormalities and infection following severe thermal injury. Ann. Surg. 188: 809-816.

Alverdy,J.C. 1990. Effects of Glutamine-Supplemented Diets on Immunology of the Gut. Journal of Parenteral and Enteral Nutrition 14: 109S-113S.

Ardawi,M.S. and Newsholme,E.A. 1983. Glutamine metabolism in lymphocytes of the rat. Biochem. J. 212: 835-842.

Armstrong,C.P., Taylor,T.V., and Torrence,H.B. 1987. Ionic flux and mucosal ultrastructure in the rat bile-pancreatic duct. Effect of bile salt perfusion on duct integrity. Dig. Dis. Sci. 32: 861-871.

Arturson,G. 1985. Neutrophil granulocyte functions in severely burned patients. Burns Incl. Therm. Inj. 11: 309-319.

Basoglu,M., Yildirgan,I., Akcay,F., Kiziltunc,A., Kavak,I., and Oren,D. 1997. Glutathione and nitric oxide concentrations in glutamine-infused rabbits with intestinal ischaemia/reperfusion. Eur. J. Clin. Chem. Clin. Biochem. 35: 415-419.

Bjorksten,B. 1999. Environment and infant immunity. Proc. Nutr. Soc. 58: 729-732.

Bozic,F., Bilic,V., and Valpotic,I. 2002a. Modulation by levamisole of CD45RA and CD45RC isoforms expression in the gut of weaned pigs vaccinated against colibacillosis. J. Vet. Pharmacol. Ther. 25: 69-72.

Bozic,F., Lackovic,G., Stokes,C.R., and Valpotic,I. 2002b. Recruitment of intestinal CD45RA+ and CD45RC+ cells induced by a candidate oral vaccine against porcine post-weaning colibacillosis. Vet. Immunol. Immunopathol. 86: 137-146.

Braakman,E., Sturm,E., Vijverberg,K., van Krimpen,B.A., Gratama,J.W., and Bolhuis,R.L. 1991. Expression of CD45 isoforms by fresh and activated human gamma delta T lymphocytes and natural killer cells. Int. Immunol. 3: 691-697.

Chang,W.K., Yang,K.D., and Shaio,M.F. 1999. Effect of glutamine on Th1 and Th2 cytokine responses of human peripheral blood mononuclear cells. Clin. Immunol. 93: 294-301.

Chertov,O., Yang,D., Howard,O.M., and Oppenheim,J.J. 2000. Leukocyte granule proteins mobilize innate host defenses and adaptive immune responses. Immunol. Rev. 177: 68-78.

DeMarco,V.G., Li,N., Thomas,J., West,C.M., and Neu,J. 2003. Glutamine and barrier function in cultured Caco-2 epithelial cell monolayers. J. Nutr. 133: 2176-2179.

Duffey,M.E., Hainau,B., Ho,S., and Bentzel,C.J. 1981. Regulation of epithelial tight junction permeability by cyclic AMP. Nature 294: 451-453.

Dugan,M.E. and McBurney,M.I. 1995. Luminal glutamine perfusion alters endotoxin-related changes in ileal permeability of the piglet. JPEN J. Parenter. Enteral Nutr. 19: 83-87.

Egberts,H.J., de Groot,E.C., van Dijk,J.E., Vellenga,L., and Mouwen,J.M. 1993. Tight junctional structure and permeability of porcine jejunum after enterotoxic Escherichia coli infection. Res. Vet. Sci. 55: 10-14.

Faurschou,M. and Borregaard,N. 2003. Neutrophil granules and secretory vesicles in inflammation. Microbes. Infect. 5: 1317-1327.

Field,C.J., Johnson,I., and Pratt,V.C. 2000a. Glutamine and arginine: immunonutrients for improved health. Med. Sci. Sports Exerc. 32: S377-S388.

Field,C.J., Thomson,C.A., Van Aerde,J.E., Parrott,A., Euler,A., Lien,E., and Clandinin,M.T. 2000b. Lower proportion of CD45R0+ cells and deficient interleukin-10 production by formula-fed infants, compared with human-fed, is corrected with supplementation of long-chain polyunsaturated fatty acids. J. Pediatr. Gastroenterol. Nutr. 31: 291-299.

Field,C.J., Van Aerde,J.E., Robinson,L.E., and Clandinin,M.T. 2003. Effect of feeding long chain polyunsaturated fatty acids to term infants over the first 6 weeks of life on the response to mitogens and food proteins. Exp. Biol. Abstract # 7150.

Fletcher,J., Haynes,A.P., and Crouch,S.M. 1990. Acquired abnormalities of polymorphonuclear neutrophil function. Blood Rev. 4: 103-110.

Fukatsu,K., Ueno,C., Hashiguchi,Y., Hara,E., Kinoshita,M., Mochizuki,H., and Hiraide,H. 2003. Glutamine infusion during ischemia is detrimental in a murine gut ischemia/reperfusion model. JPEN J. Parenter. Enteral Nutr. 27: 187-192.

Furukawa,S., Saito,H., Fukatsu,K., Hashiguchi,Y., Inaba,T., Lin,M.T., Inoue,T., Han,I., Matsuda,T., and Muto,T. 1997. Glutamine-enhanced bacterial killing by neutrophils from postoperative patients. Nutrition 13: 863-869.

Furukawa,S., Saito,H., Inoue,T., Matsuda,T., Fukatsu,K., Han,I., Ikeda,S., and Hidemura,A. 2000. Supplemental glutamine augments phagocytosis and reactive oxygen intermediate production by neutrophils and monocytes from postoperative patients in vitro. Nutrition 16: 323-329.

Gennari,R. and Alexander,J.W. 1997. Arginine, glutamine, and dehydroepiandrosterone reverse the immunosuppressive effect of prednisone during gut-derived sepsis. Crit Care Med. 25: 1207-1214.

Guandalini,S., Rao,M.C., Smith,P.L., and Field,M. 1982. cGMP modulation of ileal ion transport: in vitro effects of Escherichia coli heat-stable enterotoxin. Am. J. Physiol 243: G36-G41.

Gyles,C.L. 1992. Escherichia coli cytotoxins and enterotoxins. Can. J. Microbiol. 38: 734-746.

Hamann,D., Baars,P.A., Rep,M.H., Hooibrink,B., Kerkhof-Garde,S.R., Klein,M.R., and van Lier,R.A. 1997. Phenotypic and functional separation of memory and effector human CD8+ T cells. J. Exp. Med. 186: 1407-1418.

Haverson,K., Bailey,M., and Stokes,C.R. 1999. T-cell populations in the pig intestinal lamina propria: memory cells with unusual phenotypic characteristics. Immunology 96: 66-73.

Horig,H., Spagnoli,G.C., Filgueira,L., Babst,R., Gallati,H., Harder,F., Juretic,A., and Heberer,M. 1993. Exogenous glutamine requirement is confined to late events of T cell activation. J. Cell Biochem. 53: 343-351.

House,J.D., Pencharz,P.B., and Ball,R.O. 1994. Glutamine supplementation to total parenteral nutrition promotes extracellular fluid expansion in piglets. J. Nutr. 124: 396-405.

Ikeda,S., Kudsk,K.A., Le,T., Zarzaur,B.L., and Johnson,C.D. 2003. Glutamine improves impaired cellular exudation and polymorphonuclear neutrophil phagocytosis induced by total parenteral nutrition after glycogen-induced murine peritonitis. Shock 19: 50-54.

Isolauri,E., Kaila,M., Arvola,T., Majamaa,H., Rantala,I., Virtanen,E., and Arvilommi,H. 1993. Diet during rotavirus enteritis affects jejunal permeability to macromolecules in suckling rats. Pediatr. Res. 33: 548-553.

Jensen,G.L., Miller,R.H., Talabiska,D.G., Fish,J., and Gianferante,L. 1996. A double-blind, prospective, randomized study of glutamine-enriched compared with standard peptide-based feeding in critically ill patients. American Journal of Clinical Nutrition 64: 615-621.

Jung,T., Schauer,U., Heusser,C., Neumann,C., and Rieger,C. 1993. Detection of intracellular cytokines by flow cytometry. J. Immunol. Methods 159: 197-207.

Kanegane,H., Kasahara,Y., Niida,Y., Yachie,A., Sughii,S., Takatsu,K., Taniguchi,N., and Miyawaki,T. 1996. Expression of L-selectin (CD62L) discriminates Th1- and Th2-like cytokine-producing memory CD4+ T cells. Immunology 87: 186-190.

Keljo,D.J., Butler,D.G., and Hamilton,J.R. 1985. Altered jejunal permeability to macromolecules during viral enteritis in the piglet. Gastroenterology 88: 998-1004.

Kew,S., Wells,S.M., Yaqoob,P., Wallace,F.A., Miles,E.A., and Calder,P.C. 1999. Dietary glutamine enhances murine T-lymphocyte responsiveness. J. Nutr. 129: 1524-1531.

Klimberg,V.S., Salloum,R.M., Kasper,M., Plumley,D.A., Dolson,D.J., Hautamaki,R.D., Mendenhall,W.R., Bova,F.C., Bland,K.I., Copeland,E.M., III, and Souba,W.W. 1990. Oral Glutamine Accelerates Healing of the Small Intestine and Improves Outcome After Whole Abdominal Radiation. Archives of Surgery 125: 1040-1045.

Kohler,H., Hartig-Knecht,H., Ruggeberg,J., Adam,R., and Schroten,H. 2000. Lymphocyte proliferation is possible with low concentrations of glycyl-glutamine. Eur. J. Nutr. 39: 103-105.

Kuhn,K.S., Stehle,P., and Fürst,P. 1996. Glutamine content of protein and peptide-based enteral products. Journal of Parenteral and Enteral Nutrition 20: 292-295.

Liu,C.T., Chen,K.M., Chang,P.L., and Lii,C.K. 2001. Glutamine utilization in activated lymphocytes from rats receiving endotoxin. J. Surg. Res. 96: 246-254.

Mascher,B., Schlenke,P., and Seyfarth,M. 1999. Expression and kinetics of cytokines determined by intracellular staining using flow cytometry. J. Immunol. Methods 223: 115-121.

Mohanty,P., Ghanim,H., Hamouda,W., Aljada,A., Garg,R., and Dandona,P. 2002. Both lipid and protein intakes stimulate increased generation of reactive oxygen species by polymorphonuclear leukocytes and mononuclear cells. Am. J. Clin. Nutr. 75: 767-772.

Moritz,A., Walcheck,B.K., Deye,J., and Weiss,D.J. 2003. Effects of short-term racing activity on platelet and neutrophil activation in dogs. Am. J. Vet. Res. 64: 855-859.

Murphy,C. and Newsholme,P. 1998. Importance of glutamine metabolism in murine macrophages and human monocytes to L-arginine biosynthesis and rates of nitrite or urea production. Clin. Sci. 95: 397-407.

Nabuurs,M.J. 1998. Weaning piglets as a model for studying pathophysiology of diarrhea. Vet. Q. 20 Suppl 3: S42-S45.

Newsholme,E.A. and Calder,P.C. 1997. The proposed role of glutamine in some cells of the immune system and speculative consequences for the whole animal. Nutrition 13: 728-730.

Newsholme,E.A., Crabtree,B., and Ardawi,M.S. 1985. The role of high rates of glycolysis and glutamine utilization in rapidly dividing cells. Biosci. Rep. 5: 393-400.

Newsholme,E.A. and Parry-Billings,M. 1990. Properties of Glutamine Release From Muscle and Its Importance for the Immune System. Journal of Parenteral and Enteral Nutrition 14: 63S-67S.

NRC 1998. Nutrient Requirements of swine (10th Ed.). Subcom. Swine Nutr., Comm Anim. Nutr., Board Agric., NRC. Nat. Acad. Press, Wash. pp 192.

Nusrat,A., Turner,J.R., and Madara,J.L. 2000. Molecular physiology and pathophysiology of tight junctions. IV. Regulation of tight junctions by extracellular stimuli: nutrients, cytokines, and immune cells. Am. J. Physiol Gastrointest. Liver Physiol 279: G851-G857.

O'Dowd,Y. and Newsholme,P. 1997. Evidence for the involvement of glutamine in nitric oxide (NO) production by immunostimulated neutrophils. Biochem. Soc. Trans. 25: 403S.

Ogle,C.K., Ogle,J.D., Mao,J.-X., Simon,J., Noel,J.G., Li,B.-G., and Alexander,J.W. 1994. Effect of Glutamine on Phagocytosis and Bacterial Killing by Normal and Pediatric Burn Patient Neutrophils. Journal of Parenteral and Enteral Nutrition 18: 128-133.

Panigrahi,P., Gewolb,I.H., Bamford,P., and Horvath,K. 1997. Role of glutamine in bacterial transcytosis and epithelial cell injury. JPEN J. Parenter. Enteral Nutr. 21: 75-80.

Parry-Billings,M., Evans,J., Calder,P.C., and Newsholme,E.A. 1990. Does glutamine contribute to immunosuppression after major burns? Lancet 336: 523-525.

Pithon-Curi,T.C., De Melo,M.P., De Azevedo,R.B., Zorn,R.M.T., and Curi,R. 1997. Glutamine utilization by rat neutrophils: presence of phosphate-dependent glutaminase. Am. J. Physiol. 273 (Cell Physiol.): C1124-C1129.

Pithon-Curi,T.C., Levada,A.C., Lopes,L.R., Doi,S.Q., and Curi,R. 2002a. Glutamine plays a role in superoxide production and the expression of p47phox, p22phox and gp91phox in rat neutrophils. Clin. Sci. (Lond) 103: 403-408.

Pithon-Curi,T.C., Trezena,A.G., Tavares-Lima,W., and Curi,R. 2002b. Evidence that glutamine is involved in neutrophil function. Cell Biochem. Funct. 20: 81-86.

Rhoads,J.M., Keku,E.O., Bennett,L.E., Quinn,J., and Lecce,J.G. 1990. Development of L-glutamine-stimulated electroneutral sodium absorption in piglet jejunum. Am. J. Physiol 259: G99-107.

Rhoads,J.M., Keku,E.O., Quinn,J., Woosely,J., and Lecce,J.G. 1991. L-glutamine stimulates jejunal sodium and chloride absorption in pig rotavirus enteritis. Gastroenterology 100: 683-691.

Rhoads,J.M., Keku,E.O., Woodard,J.P., Bangdiwala,S.I., Lecce,J.G., and Gatzy,J.T. 1992. L-glutamine with D-glucose stimulates oxidative metabolism and NaCl absorption in piglet jejunum. Am. J. Physiol 263: G960-G966.

Rodriguez-Carreno,M.P., Lopez-Fuertes,L., Revilla,C., Ezquerra,A., Alonso,F., and Dominguez,J. 2002. Phenotypic characterization of porcine IFN-gamma-producing lymphocytes by flow cytometry. J. Immunol. Methods 259: 171-179.

Rohde,T., MacLean,D.A., and Klarlund,P.B. 1996. Glutamine, lymphocyte proliferation and cytokine production. Scand. J. Immunol. 44: 648-650.

Ruggeberg,J., Stalmach,E., Zubrod-Eichert,C., Wahn,V., and Schroten,H. 1997. Antimicrobial Functions of Human Monocytes Depend on Concentration of glutamine in vitro. Annals of Nutrition Metabolism 41: 371-375.

Shewchuk,L.D., Baracos,V.E., and Field,C.J. 1997. Dietary L-glutamine supplementation reduces the growth of the Morris Hepatoma 7777 in exercise-trained and sedentary rats. J. Nutr. 127: 158-166.

Stasny,B.R., De Guise,S., Rompato,G., and Garmendia,A.E. 2001. Functional characterization of a swine CD4(+)/CD8(+) double positive lymphoblastoid T-cell line with a CD25(+)/CD45RA(-) phenotype generated in vitro with interleukin-2. Vet. Immunol. Immunopathol. 78: 57-70.

Suzuki,I., Matsumoto,Y., Adjei,A.A., Asato,L., Shinjo,S., and Yamamoto,S. 1993. Effect of a glutamine-supplemented diet on response to methicillin-resistant Staphylococcus aureus infection in mice. J. Nutr. Sci. Vitaminol. (Tokyo) 39: 405-410.

Swaisgood,H.E. 1982. Chemistry of milk protein. In Developments in dairy chemistry. Fox,P.F., ed. Applied Science Publishers, London, pp 1-61.

Szabo,S.J., Sullivan,B.M., Peng,S.L., and Glimcher,L.H. 2003. Molecular mechanisms regulating Th1 immune responses. Annu. Rev. Immunol. 21: 713-758.

van Acker,B.A., von Meyenfeldt,M.F., van der Hulst,R.R., Hulsewe,K.W., Wagenmakers,A.J., Deutz,N.E., de,B., I, Dejong,C.H., van Kreel,B.K., and Soeters,P.B. 1999. Glutamine: the pivot of our nitrogen economy? JPEN J Parenter. Enteral Nutr 23: S45-S48.

van der Hulst,R.R., von Meyenfeldt,M.F., Deutz,N.E., Stockbrugger,R.W., and Soeters,P.B. 1996. The effect of glutamine administration on intestinal glutamine content. J. Surg. Res. 61: 30-34.

Vlessis,A.A., Goldman,R.K., and Trunkey,D.D. 1995. New concepts in the pathophysiology of oxygen metabolism during sepsis. Br. J. Surg. 82: 870-876.

Weiss,D.J. and Evanson,O.A. 2003. Evaluation of activated neutrophils in the blood of horses with colic. Am. J. Vet. Res. 64: 1364-1368.

Woodward,B.D., Bezanson,K.D., Hillyer,L.M., and Lee,W.H. 1995. The CD45RA+ (quiescent) cellular phenotype is overabundant relative to the CD. J. Nutr. 125: 2471-2482.

Wu,G. 1996. An important role for pentose cycle in the synthesis of citrulline and proline from glutamine in porcine enterocytes. Arch. Biochem. Biophys. 336: 224-230.

Wu,G. 1998. Intestinal mucosal amino acid catabolism. J Nutr 128: 1249-1252.

Yalçin,S.S., Yurdakök, K., Tezcan,I., and Öner,L. 2004. Effect of glutamine supplementation on diarrhea, interleukin-8 and secretory immunoglobulin A in children with acute diarrhea. J. Ped. Gastr. Nutr. 38: 494-501.

Yaqoob,P. and Calder,P.C. 1998. Cytokine production by human peripheral blood mononuclear cells: differential sensitivity to glutamine availability. Cytokine 10: 790-794.

Yoo,S.S., Field,C.J., and McBurney,M.I. 1997. Glutamine supplementation maintains intramuscular glutamine concentrations and normalizes lymphocyte function in infected early weaned pigs. J Nutr 127: 2253-2259.

Chapter 6 – INFLUENCE OF BARLEY-DERIVED BETA-GLUCANS ON SWINE IMMUNE AND GUT DEVELOPMENT

6.1 INTRODUCTION

Weaning in young animals and humans is associated with an increased incidence of gastrointestinal infections. Non-fermentable dietary fiber promotes normal colonic function by increasing fecal weight and bowel frequency (Slavin et al. 1985). Furthermore, the bacterial metabolism of dietary fibers or other fermentable substrates, such as fructooligosaccharides, to short-chain fatty acids (SCFA), is essential in maintaining small bowel and colonic mucosal structure and function (Buddington 1998). Although relatively little work has been done in pediatric populations (Correa-Matos et al. 2003), a study found that feeding soy polysaccharide reduced the duration of bacterial and viral diarrhea in developing countries (Brown et al. 1993). In addition, the use of soy polysaccharide-supplemented formula was reported to reduce the duration of diarrheal symptoms in U.S. infants greater than 6 months old with acute diarrhea (Vanderhoof et al. 1997). Thus there is physiologic rationale to support the addition of dietary fiber to infant formulas (Correa-Matos et al. 2003).

Barley 1,3/1,4 beta-glucans (B-glc) is the major component of barley endosperm cells walls, accounting for about 75% (w/w) of total cell-wall carbohydrates, the remainder of which is protein and pentosans (Jiang & Vasanthan 2000). B-glc from barley consists of a linear chain of glucose residues, made up of approximately 70% B(1-4) and 30% B(1-3) glycosidic links (Jiang & Vasanthan 2000). Providing B-glc in the diet is reported to increase fecal bulk, relieving constipation (Izydorczyk et al. 2000), to lower postprandial plasma glucose and insulin concentrations (Braaten et al. 1991), to increase bile acid excretion (Judd & Truswell 1981; Kirby et al. 1981; Anderson et al. 1984; Jenkins et al. 1993) and to alter the microflora and production of SCFAs (Dongowski et al. 2002). In addition, oat derived B-glc (Anderson et al. 1984; Braaten et al. 1994; Lia et al. 1995; Zhang et al. 1992), as well as wheat- and barley-derived B-glc (McIntosh et al. 1991) have been associated with reductions in serum cholesterol concentrations in both human and animals.

The ability of B-glc to influence immune function *in vitro* was first demonstrated over 40 yrs ago (Riggi & Di Luzio 1961) and the effect appears to be dependent on the size of the B-glc particles. While very short B-glc (<5000-10,000 molecular weight) are generally considered inactive, large molecular weight B-glc have been demonstrated to directly activate leukocytes *in vitro* (Brown & Gordon 2003) by binding to receptors (i.e. CR3, lactosylceramide, Dectin-1 (Brown et al. 2003)). A role of B-glc in bacterial defense has also been demonstrated *in vivo*. Intravenous injections of glucans derived from *Saccharomyces cerevisiae* were shown to promote resistance to *Staphylococcus aureus* infections in normal, leukemic as well as immunosuppressed mice (Williams & Di Luzio 1979). Furthermore, a single intraperitoneal administration of oat-derived B-glc enhanced the survival of mice challenged with *Staphylococcus aureus* (Estrada et al. 1997). The mechanism for this effect is not known but could be related to the effect of B-glc on immune cell populations. An increased proportion of murine polymorphonuclear neutrophils (neutrophils) and eosinophils were reported following intraperitoneal injection of yeast-derived B-glc (Cleary et al. 1999) and an accumulation of macrophages in the peritoneal cavity following intraperitoneal administration of oat-derived B-glc (Estrada et al. 1997). In humans, an increased proportion of human peritoneal macrophages was found following combined immunotherapy (sizofiran beta-1,3-glucan & rIFN-γ) (Chen & Hasumi 1993). Suppression of tumor growth has also been produced by *in vivo* injection of mice with glucans from several sources (Schizophyllum commune (Komatsu et al. 1969), *Saccharomyces cerevisiae* (Di Luzio et al. 1979b; Di Luzio et al. 1979a; Sherwood et al. 1988)) and lentinan (B-glc from mushrooms) has been used in human cancer therapy (reviewed in (Chihara 1992)). Whether the B-glc anti-tumor action is due to their effect on immune function is not clear from these studies.

B-glc (purified mushroom polysaccharide) polymers have been shown to influence acquired immune function *in vitro* by increasing lymphocyte proliferation and cytotoxicity (Kim et al. 1996). Intravenous administration of B-glc (derived from Sclerotinia sclerotiorum) to mice resulted in activation of lymphocytes and alveolar macrophages, demonstrated by increased IFN-γ concentrations in bronchoalveolar lavage fluid, as well as increased inflammatory cytokine (Interleukin (IL)-1, IL-6, TNF-α) mRNAs in lung tissue (Sakurai et al. 1994). Intravenous injection of soluble glucan has also been shown to enhance murine splenic macrophage IL-1 production and splenic lymphocyte IL-2 production (Sherwood et al. 1987). In patients with gynecological malignancies, peritonaeal injection of b-1,3-glc sizofiran and rIFN-γ increased the production of IL-1β, IFN-γ and TNF-α (Chen & Hasumi 1993). *In vitro* incubation of oat-derived B-glc stimulated the production of cytokines (IL-2, IFN-γ and IL-4) in murine cultured spleen cells and IL-1 in cultured macrophages (Estrada et al. 1997). Fungal B-glc stimulate murine peritoneal macrophage production of TNF-α and IL-1 (Hashimoto et al. 1997) and stimulate TNF-α release from alveolar macrophages of rats (Hoffman et al. 1993) and rabbits (Olson et al. 1996). In humans, exposure of peripheral blood monocytes to aminated B-glc (water-soluble derivative prepared by reductive amination) induced secretion of TNF-α and IL-1β (Doita et al. 1991). Furthermore, attachment of high molecular weight or particulate B-glc to macrophage and neutrophil complement CR3 stimulates the secretion of a broad host of cytokines (i.e. IL-1, IL-6, IL-8, TNF-α, IFN-α and IFN-γ) (Rasmussen et al. 1987; Rankin et al. 1990; Abel & Czop 1992; Au et al. 1994; Ohno et al. 1996).

Barley is used only to a small extent for human food in most developed countries. It is predominantly used in the brewing industry (Dongowski et al. 2002) and in animal diets, such as swine. Despite the evidence that some B-glc can modulate immune function (at least *in vitro*), the extent to which B-glc influence piglet immune function is unknown (Hiss & Sauerwein 2003) and the translation of these results to the consumption of b-glc obtained in the diet from cereals, particularly barley, is unknown. Therefore, the objectives of this study were to determine the effect of feeding barley derived b-glc on immune and gastrointestinal function. It was hypothesized that supplementation of barley-derived B-glc will improve innate immune defense (neutrophil function), improve adaptive immune defense and benefit gut development in newly weaned piglets. Supplementation of diets varying in fiber and B-glc composition to piglets was also hypothesized to decrease the ability of isolated enterocytes to bind fluorescein isothiocyanate (FITC)-labelled *Escherichia coli* (*E. coli*).

6.2 MATERIALS & METHODS

Animals and Diets

Animal protocols were reviewed and approved by the Faculty of Agriculture and Forestry Animal Policy and Welfare Committee and were conducted in accordance with the Canadian Council on Animal Care guidelines. Thirteen male and eighteen female Dutch-Landrace (Genex Swine Group, Heartland Livestock Services, Regina, Saskatchewan) piglets were obtained from the University of Alberta Swine Research and Technology Centre and transported to a separate, temperature-controlled room at the University of Alberta Metabolic Research Unit. Piglets were housed individually in metabolic crates, each fitted with water nipples and creep feeders. Body weight (BW) and feed intake (FI) were recorded daily during the feeding trial.

Piglets from each litter (n = 31, 4 litters) were obtained at approximately 21 days of age, and weaned onto the experimental diets. The B-glc feeding design is shown in the Appendix (Figure 3) and diets were formulated to meet 110% of the nutrient requirements for piglet 5-10 kg as specified by the National Research Council (NRC 1998). A constant portion of the diet contained 100 g/kg of fat from a mixture of sources (59.7% Tallow, 33.1% Safflower Oil, and 7.2% Linseed Oil), providing a polyunsaturated to saturated fatty acid ratio of ~ 0.9. The weaning diet compositions are given in Table 1 and consist of: 1) standard diet with wheat as main feedstuff 2) standard weaning diet with Barley 3) standard weaning diet with Candle barley Cultivar (provided by Dr. B.G. Rossnagel, University of Saskatchewan) and 4) standard diet with Candle barley Cultivar with added Candle Fiber (Viscofiber^{TM}, Cevena Bioproducts, Edmonton, Alberta). This waxy barley cultivar was selected because of its high B-glc content, which was previously reported to be 6.89% (w/w) total B-glc (Izydorczyk et al. 2000). The supplement portion of the diet (70 g/kg) was Candle Fiber (Viscofiber^{TM}) for diet 4, and was balanced in the remaining three diets using casein (4.82 g/kg, Harlan Teklab Test Diets) as the protein source, Candle Crude Starch (12.4 g/kg) and non-nutritive cellulose (52.8 g/kg, Harlan Teklab Test Diets) as the fiber source. Each diet was designed to give an increasing amount of total B-glc (Diet 1 = 0.642%, Diet 2 = 1.73%, Diet 3 = 3.55%, Diet 4 = 7.37%). The chemical composition of the major dietary ingredients was analyzed and is resented in Table 2.

Sample Collection

At approximately 35 days of age, samples were collected for study of immune function and gut development as previously described (Chapter 4) with the following modifications. The entire small intestine was removed from each piglet (from ligament of Treitz to ileo-cecal junction) by dissecting away from the mesentery. Each section was then flushed (approximately 30 mls Phosphate-Buffered Saline (PBS)) and smoothed to remove intestinal contents. Wet weight and total length were then recorded. Intestinal sections (20-30 cm) from the jejunum (proximal 20-30 cm), promixal ileum (20-30 cm at exactly the midpoint of the small intestine) and distal ileum (distal 20-30 cm from 10 cm section for Peyer's Patch (PP) isolation) were cut, tied off at one end (umbilical tape) and stored in separate containers containing ice-cold PBS for subsequent isolation of mucosal cells. For Ussing chamber measurements, a jejunal (approximately 10 cm, distal to section obtained for enterocyte isolation) and ileal section (approximately 10 cm, proximal to section obtained for enterocyte isolation) were cut, tied at one end, and distended with ice-cold PBS. The loop was then closed, immersed in a 50 ml sterile culture tubes containing ice-cold PBS and stored immediately on ice.

Blood Chemistry

Whole blood samples were collected in 3 ml Vacutainer tubes (Lavender conventional closure for whole blood hematology determination, Becton Dickinson, Franklin Lanes, New Jersey) for blood chemistry assessment. Samples were auto-analyzed using the Coulter AC.T 5diff CP (Beckman Coulter, Fullerton, California).

Immune Measures

As previously described and under sterile conditions, peripheral blood lymphocytes (PBL) and immune cells from mesenteric lymph nodes (MLN) (Chapter 3), as well as from PP (Chapter 4) were isolated. Incorporation of ^{3}H-thymidine was determined as previously described (Chapter 3). Immune cells were incubated with or without the mitogens PHA (25 *u*g/ml; Sigma), PMAI (PMA 20 ng/ml, Ionomycin 0.5 nmol/L), PWM (55 *u*g/ml; Sigma) and LPS (1 mg/ml; Sigma). Additionally, production of cytokines by immune cells from blood and MLN was determined as previously described (Chapter 4). Isolated cells from each immune tissue (PBL & MLN) were stimulated PHA (25 *u*g/ml), PMAI (PMA 20 ng/ml, Iono 0.5 nmol/L), PWM (55 *u*g/ml) and LPS (1 mg/ml) and the production of IL-2, IL-4, IL-10, IFN-γ and TNF-α was measured. Lymphocyte subsets from freshly isolated immune cells in blood, PP and MLN were identified by indirect immunofluorescence assay as previously described (Chapter 3). The monoclonal antibodies (mAb) combinations used in these studies is provided in the Appendix. As described previously, whole blood was used to assess neutrophil function (Chapter 3).

Ussing Chambers

Distended intestinal sections were transported to the Ussing Chamber Unit (6th Floor, Dentistry/Pharmacy Building) on ice. Tissues were mounted in Ussing chambers and measurements were performed (Mannitol permeability (ManP), Short-circuit current (Isc), Potential Difference (PD), Forskolin (Fors) and Conductance (G)) as described previously (Chapter 3).

Adhesion Assay

Mucosal cells were isolated from each gut section (jejunum, proximal & distal Ileum) according to methods described elsewhere (Fan et al. 2001). Briefly, using 30 ml syringes (Becton Dickinson) and needles (18G1, Becton Dickinson), intestinal segments tied at one end and filled with a preincubation buffer (sodium citrate (27 mM; Sigma Chemical, Oakville, ON), phenylmethylsulfonyl fluoride (PMSF; 0.2 mM; Sigma), and dithiothreitol (DTT; 0.5 mM; Sigma), ph 7.4, oxygenated with O_2/CO_2 mixture 19:1 vol/vol), then sealed with hemostatic clamps. Sections were immersed in oxygenated, pre-warmed (37°C) saline (154 mM; Sigma) and 2 L glass trays and lightly agitated for approximately 15 min. This preincubation wash was subsequently discarded and segments were re-filled with an isolation buffer (Na_2- ethylenediaminetetraacetic acid

(EDTA; 1.5 mM; Sigma), PMSF (0.2 mM; Sigma), DTT (0.5 mM; Sigma), D-glucose (5mM; Sigma), oxygenated with O_2/CO_2 mixture), sealed and immersed in warm, oxygenated saline with light agitation for approximately 15 min. Mucosal cells obtained from this second wash were collected and washed once with PBS. Mucosal cells from the distal ileum were re-suspended in 30% Percoll (10 mls w/w). This mixture was gently transferred over 70% Percoll (10 mls w/w) contained in sterile culture tubes. Tubes were spun at 700 x g for 40 min. (no brake). The enterocyte band was then removed, transferred to a fresh 50 ml Falcon tube (Fischer), and re-suspended in PBS (with bovine serum albumin (BSA; 0.25%; Sigma), D-glucose (4 mM; Sigma) and glutamine (gln; 2 mM; Sigma). This final cell suspension was counted by Trypan Blue exclusion and stage microscospy. Approximately 5.0×10^5 mucosal cells from each segment were added to FACSCAN tubes (Becton Dickinson) for the subsequent use in the *E. coli* binding assay.

Fresh cultures of K88ac ETEC (kindly provided by Dr. Marquardt, University of Manitoba, Winnipeg, MB, Canada) were prepared by transferring a small sample of ETEC frozen culture to 6 mls of BHI media (Oxoid LTD., Basingstoke, Hampshire, England) and growing for 24 hrs (in shaking incubator at 37^oC). This ETEC culture was subsequently subcultured (24 hrs). The final culture was prepared by transfer of 2 mls of subcultured ETEC media to a 200 ml flask with BHI media (Oxoid) and grown in a shaking incubator at 37^0C for approximately 6 hrs. At this time, duplicate samples (10 mls) were obtained from the flask. The first sample was labeled with FITC as previously described (F-143 'Isomer I', Molecular Probes, Inc.; (Clarke & Morton 2000)). The second sample was used for determination of the bacterial culture concentration by recording absorbance (Spectrophotometer, OD 595 nm) and verifying the concentration on the ETEC growth curve (see Figure 4 of APPENDIX for growth curve). For each culture, the presence of K88 fimbriae was confirmed by a commercial latex agglutination test (VETWEY FIMBREX K88, Central Veterinary Laboratory, Surrey, UK).

The ability of enterocytes to bind *E.coli* was assessed by flow cytometry. FACSCAN tubes containing isolated mucosal cells and separate tubes containing FITC-labeled ETEC were transported to the FACSCAN unit (University of Alberta Heritage Building). Using acquisition software (SSC=345, FL1=329), FITC-ETEC and mucosal cells controls were measured before commencing the adhesion assay. Subsequent measurements were taken immediately following addition of FITC-ETEC (calculated as 1.0 x 10^7 CFU/ml for each reaction) to tubes containing mucosal cells (Time 0) and at 5 subsequent time points (Time 1 – 5 min, approximately 20,000 gated events) (Figure 1). Fluorescence intensity for each cell, indicative of the amount of bound FITC-labeled ETEC was measured by emission of FITC fluorescence light excited by the FACScan laser. To verify maximum binding, an additional timed control was measured containing mucosal cells combined with FITC-ETEC for an extended time period (approximately 20-49 min.).

Statistics

All statistical analyses were completed using the SAS statistical package (Version 8.1, SAS Institute, Cary, NC). Results were presented as means ± SEM. For BW and FI, neutrophil assay, proliferation and phenotype, the effect of diet was determined by one-way ANOVA, blocked for litter. Adhesion assay data was analyzed with a repeated measures design. Kurtosis and skew were analyzed for proliferation and cytokine data. Values not considered normally distributed by kurtosis and skew were log transformed prior to statistical analysis and are indicated as log transformed in their respective tables. Immune and gut measures were also tested for linearity and quadratic relationships. Following the input for each analysis parameter (i.e. data = blood chemistry), this was accomplished by defining the level of B-glc (data = bglc) for each parameter that was obtained (merge blood chemistry bglc). Linear and quadratic relationships were determined by using proc glm with model y = B-glc B-glc*B-glc block/solution.

6.3 RESULTS

Weight and Feed Intake

There were no differences in initial or final BW or average daily gain (ADG) (Table 3). Wheat-fed piglets consumed significantly more diet (FI, g/d & g/kg BW) than piglets fed Barley, Candle, and B-glc diets. Diet did not influence piglet intestinal wet weight or the length of the small intestine (Table 3).

Blood Chemistry

Blood chemistry analysis revealed no significant effect of diet on the concentration of hemoglobin (g/L), mean corpuscular hemoglobin concentration (g/L), mean corpuscular hemoglobin (pg), or platelets (10^9/L), on the hematocrit (L/L), or mean corpuscular- (fL) or mean platelet volumes (fL), on the percentage of red cell distribution width, neutrophils, lymphocytes, monocytes, eosinophils, or basophils, or the numbers of neutrophils, monocytes, or basophils (10^9/L) (Table 4). The blood chemistry values obtained in this study were within normal ranges, with the exception of eosinophils, which were found to be lower than the normal range in each of the diet groups studied (Table 4). Piglets fed Barley had a significantly higher concentration of white blood cells (10^9/L) and esosinophils (10^9/L) than wheat fed-piglets (Figure 2). B-glc-fed piglets had a significantly higher concentration of red blood cells (10^{12}/L) than barley-fed piglets and significantly higher concentration of white blood cells (10^9/L) than Candle-fed piglets (Figure 2). The increase in white blood cells was likely due to an increase in the concentration of lymphocytes (10^9/L) in B-glc fed piglets (Figure 2). There was a direct linear relationship between dietary B-glc content and the concentration of red blood cells ($p<0.005$), the hematocrit ($p<0.05$), and the lymphocyte concentration ($p<0.02$). In B-glc-fed piglets, there was a significantly increased concentration of CD4 and CD45RA in blood (Table 5). This increase was illustrated by a linear relationship between dietary B-glc and the concentration of CD4 ($p<0.005$), CD45RA ($p<0.005$), and CD8 ($p<0.05$).

Neutrophil Function

Neutrophil burst, size and granularity did not differ significantly amongst diets (Table 6).

Immunofluorescence

T cell distribution in PBL (Table 7), MLN (Table 8) and PP (Table 9) of CD2, CD3, CD4, CD8, and CD25 was not altered by changing the B-glc content of the diet. The proportion of cells in PBL, MLN and PP that were Bcell+, immunoglobulin (Ig) A+, IgM+ and IgG+, as well as the proportion of PP Bcell+ cells were not significantly changed by diet. Cells from the monocyte population were also not significantly different in PBL, MLN or PP. The proportion of cells expressing the adhesion molecules CD11b, CD44 and CDw75 was not significantly different in any of the tissues studied. The mAb (Clone # 8E6) used to detect CD3+ cells did not appear to detect CD3+ cells in this tissue (data not shown). As a consequence, it was not possible to determine the expression of the adhesion molecule CD44 on T lymphocytes. In PBL, when the percentage of T cells expressing CD45RA was calculated, there were a significantly higher percentage of CD8+ cells expressing this antigen in piglets fed the B-glc diet than in Candle-fed piglets (Figure 3). The proportion of CD8+CD45RA+ cells ($p<0.02$) and the percentage of CD8+ cells expressing CD45RA ($p<0.01$) increased in a direct relationship with the B-glc content of the diet. There was also a significant quadratic relationship in the proportion of CD2+ cells ($p<0.02$) in PBL. The proportion of CD2+ cells increased with increasing B-glc until the dietary concentration reached 35 g/kg (candle diet). Consumption of 74 g/kg (B-glc diet) resulted in a decrease in the proportion of CD2+ cells that to a similar level as the barley diet (17 g/kg B-glc). In MLN, there was a higher proportion of CD4+CD45RA+ (antigen naïve) T lymphocytes (T cells) in animals fed the barley or Candle diet, as compared to wheat (Table 8). However, the percentage of CD4+ cells in MLN expressing the CD45RA antigen was only significantly higher in Barley-fed piglets compared to wheat-fed piglets (Figure 4). There was a significant quadratic relationship in the proportion of IgG+ ($p<0.05$), CDw75+ ($p<0.05$) and CD4+CD45RA+ ($p<0.02$) in MLN. The proportion of both IgG+ and CDw75+ cells decreased with increasing B-glc until the dietary concentration reached 35 g/kg (candle diet). However, consumption of 74 g/kg (B-glc diet) resulted in an increase in the proportion of IgG+ and

CDw75+ cells that were similar to wheat diet (6.4 g/kg B-glc). The proportion of CD4+CD45RA+ cells increased with increasing B-glc until the dietary concentration reached 35 g/kg (candle diet). Consumption of 74 g/kg (B-glc diet) resulted in a decrease in the proportion of CD4+CD45RA+ cells that to a similar level as the low B-glc wheat diet. Compared to all other groups there was a higher proportion of CD4+CD45RA+ T cells in the PP of the Candle-fed groups (Figure 5), however, the proportion of T cells in this immune tissue was very low (<1% of the isolated cells) (Table 9). There was also found to be a significant quadratic relationship in the proportion of CD8-CD25+ ($p<0.02$), total CD25+ ($p<0.05$), CD4+CD45RA- ($p<0.02$), CD4+CD45RA+ ($p<0.02$) and total CD4+ ($p<0.05$) in PP. The proportion of all of these cell populations increased with increasing B-glc until the dietary concentration reached 35 g/kg (candle diet). Consumption of 74 g/kg (B-glc diet) decreased the proportion of these cell populations.

Lymphocyte Proliferation

The rate of ^{3}H-thymidine uptake by cells (from blood, MLN and PP) incubated in the absence of mitogens did not differ amongst diets (Table 10). Stimulation of immune cells from these tissues with PMAI, PHA, and PWM resulted in a rate of ^{3}H-thymidine incorporation that was not significantly influenced by diet (Table 10). However, in comparison to barley-fed piglets, PBL incubated with LPS had an increased stimulation index in the other three dietary groups (Table 10). There was a significant quadratic relationship in incorporation of ^{3}H-thymidine in unstimulated MLN cells ($p<0.05$). Proliferation of these cells increased with increasing B-glc until the dietary concentration reached 35 g/kg (candle diet). In animals consuming 74 g/kg (B-glc diet), there was a decrease in ^{3}H-thymidine incorporation by MLN cells incubated in the absence of mitogen. There was also an inverse relationship ($p<0.05$) between dietary B-glc content and ^{3}H-thymidine incorporation after PHA and PWM stimulation in PP lymphocytes.

Cytokine Production

Incubation of PBL with PHA, PMAI and LPS did not influence the production of IL-2, IL-4, IL-10, IFN-γ or TNF-α (Table 11). However, there was a significantly higher production of IL-10 by PBL of wheat-fed piglets in response to stimulation with PWM. There was a significant quadratic relationship in the production of IL-10 in response to PWM stimulation ($p<0.02$). Production of this cytokine decreased with increasing B-glc until the dietary concentration reached 35 g/kg (candle diet). Production of IL-10 increased in piglets fed 74 g/kg (B-glc diet) and was similar to IL-10 production in piglets fed 6.4 g/kg B-glc. In MLN, production of IL-2, IL-4, IL-10, IFN-γ and TNF-α was not influenced by diet, nor by the type of mitogen used (Table 12). The production of all cytokines listed in the methods was performed. Only cytokines produced following stimulation with PHA, PWM PMAI or LPS are presented in Table 11 and Table 12.

Ussing Chambers

In piglet jejunal sections, although there were no diet-induced differences in Ussing chamber measures (Table 13), there were significant linear increases in Isc ($p<0.02$) and PD ($p<0.05$) as the B-glc content of the diet increased. In the ileum, ManP (Figure 6) and G (Figure 7) were significantly higher in the piglets fed B-glc compared to all the other diets. ManP and G measures for the barley and Candle diet did not differ significantly from that of wheat (Table 13). A significant linear increase in ManP ($p<0.02$) and G ($p<0.002$) was found in ileal sections as the B-glc content of the diet increased.

Adhesion Assay

Adhesion of FITC-labeled ETEC to enterocytes was significantly influenced by diet. Compared to wheat diet, the rate of ETEC binding to isolated epithelial cells was significantly lower for cells from the Candle-fed piglets and significantly higher for piglets fed the barley diet. ETEC-binding to enterocytes from the B-glc fed piglets did not differ from wheat-fed piglets and was different from the other two groups (Figure 8).

6.4 DISCUSSION

Intake and Growth

Experimental diets were formulated to provide an equal amount of total protein (~ 26%) while increasing total B-glc content. Adjusting the dietary inclusion of B-glc with different grains resulted in differences in some other dietary components, particularly energy and fiber. Some of these differences between wheat and barley may explain why wheat-fed piglets had a slightly higher average FI (g/d & g/kg BW). However, there were no differences in final BW or ADG and diet did not influence piglet intestinal wet weight or the length of the small intestine. Previous studies have examined supplementation of B-glc derived from other sources. Similar to the current study, feeding oat-derived B-glc to piglets at higher levels (0.8-4.1%) did not negatively affect growth performance (Fortin et al. 2003). The optimal level of inclusion a commercial glucan from *Sacchromyces cerevisiae* (MacroGard-S^{TM}) (Schoenherr et al. 1994; Dritz et al. 1995; Decuypere et al. 1998) was much lower than levels used in the current study (between

0.025 and 0.05 % w/w). In these studies young pigs significantly improved daily gain (Schoenherr et al. 1994; Dritz et al. 1995), feed efficiency (Schoenherr et al. 1994), FI (Dritz et al. 1995) and final BW (28 d) (Dritz et al. 1995). More recently, supplementation of 1,3/1,6 B-glc (0.015% and 0.03%) to newly weaned piglets (4 wks) demonstrated marginal benefits for growth performance (Hiss & Sauerwein 2003).

Intestinal Permeability

The presence of indigestible saccharides in the piglet intestinal lumen has been shown to directly stimulate mucosal tissue and/or tight junctions (changes in both G and permeability) by an unknown mechanism (Mineo et al. 2002; Mineo et al. 2004). Whether enterocytes are influenced directly via the physical properties of B-glc, or indirectly, via SCFAs derived from B-glc fermentation (Velazquez et al. 1997) is unknown. Increases in distal ileal G and ManP has been shown previously in growing pigs supplemented with undigestible oligosaccharide prebiotics (topnambur powder and inactivated yeasts) (Breves et al. 2001). In the current study, ileal sections from piglets fed B-glc diets demonstrated a significantly higher ManP and G and both measures were shown to increase in a linear fashion as the B-glc content of the diet increased. Although this suggests that increasing the amount of B-glc in piglet diets may have influenced paracellular permeability, an increase in surface area would also increase manP. Therefore, changes in surface area would need to be excluded and an effect of B-glc on villus height would need to be observed before concluding that B-glc influences paracellular permeability. Increases in ManP, such as that which was shown in this study, would also be expected to increase antigen exposure and hence decrease the percentage of T cells expressing CD45RA. However this does not appear to be the case in PP and MLN cells obtained from B-glc-fed piglets, suggesting that such an increase did not change the exposure of antigens to GALT. These findings are further supported by the functional assays, in which lymphocyte incorporation of ^{3}H-thymidine *ex vivo* (unstimulated) was not significantly different among diets. Therefore, at least in terms of immune function, an increase in ManP of the distal ileum in B-glc-fed piglets does not appear to be detrimental to young piglets. Additional studies are warranted for the implications of these findings on gut development and maturation of GALT.

Piglet Immune Function

Several receptors on macrophages, other leukocytes and non-immune cells that recognize B-glc have been described (reviewed in (Brown & Gordon 2003)), but only Dectin-1 has been clearly shown to have a role in mediating the biological response to B-glc. Dectin-1 possesses a single carbohydrate recognition domain that recognizes carbohydrates containing B-1,3 and/or B-1,6 glucan linkages (Brown & Gordon 2003). B-glc receptor activity has also been reported on neutrophils, which is capable of binding B-glc, activating the neutrophil and thus triggering the oxidative burst (Brown & Gordon 2003). Improvements in neutrophil oxidative burst have been shown previously in humans (Ross et al. 1987; Zhang & Petty 1994). In this study, neutrophils isolated from healthy weaned piglets showed no diet-induced changes in neutrophil function (oxidative burst, size or granularity). This is consistent with a previous study in which pigs of a similar age fed B-glc (0.025 or 0.05% w/w) did not influence neutrophil function (Dritz et al. 1995) and supports that dietary B-glc has limited influence on neutrophil function.

It is not clear how supplementation of piglets with dietary B-glc increased the concentration of peripheral red- and white blood cells, and lymphocytes. At least for red blood cells and lymphocyte number, the increase occurred in a linear fashion. There was also a significantly increased concentration of eosinophils in barley-fed piglets. Previously, feeding fructooligosaccharides to young calves was reported to result in higher peripheral blood leukocyte numbers, specifically eosinophils (Kaufhold et al. 2000). However, these comparisons are difficult because of species differences and in the current study, the concentration of eosinophils was below the normal range (0.5-3.4 x 10^9/L; Blood & Rodostits 1989). The finding that B-glc-fed piglets had a higher concentration of peripheral lymphocytes in circulation might influence the ability of these animals to respond to immune challenge, despite no change in the function of these cells.

Previously, the fiber content of the diet has been shown to significantly alter the proportion of T cells in GALT of other species (higher proportion of CD8+ in PP (Field et al. 1999) & higher proportion of CD4+ in MLN (Field et al. 1999; Lim et al. 1997). In the current study, expression of CD45RA on T cell populations was influenced by dietary B-glc. Naïve T cells (CD4 & CD8) express the CD45RA antigen until they encounter and respond to an antigen, when results in alternative splicing of CD45 to switch to expression of CD45RO (Braakman et al. 1991). Increasing the dietary content of the B-glc was shown to increase the concentration of blood CD4 and CD45RA, as well as the percentage of CD8+ cells expressing the CD45RA antigen. There was also a direct relationship between the level of dietary B-glc and the concentration of CD4, CD8, and

CD45RA, as well as the proportion CD8+CD45RA+ cells and the percentage of CD8+ cells expressing CD45RA. Increasing the B-glc content of the diet also increased the proportion of T cells (CD2+) until the dietary concentration reached 35 g/kg (candle diet). Feeding a high level of B-glc appeared to negatively impact the proportion of T cells, as the proportion of these cells decreased to a similar level as the barley diet. These findings support the blood chemistry data and seem to suggest that a higher level of B-glc content in the diet not only increases the concentration of peripheral blood leukocytes, but specifically increases the number of naïve T cells in the periphery. In MLN, provision of 35 g/kg of B-glc appeared to be the optimal level of inclusion for the proportion of CD4+CD45RA+ cells, which increased from 6.4 to 35 g/kg. This was also the case for the proportion of CD8-CD25+, total CD25+, CD4+CD45RA-, CD4+CD45RA+, and total CD4+ in PP. Feeding a high level of B-glc appeared to negatively impact the proportion of each of these cell populations. On the other hand, increasing dietary B-glc content from 6.4 to 35 g/kg decreased the proportion of IgG+ and CDw75+ cells in MLN, whereas feeding a very high level of B-glc did not negatively impact these proportions.

A previous study conducted in newly weaned piglets reported no differences between B-glc-supplemented groups in lymphocyte proliferation or antibody titres in response to vaccination with porcine reproductive and respiratory syndrome (Hiss & Sauerwein 2003). In the current study, there was a limited influence of B-glc on ^{3}H-thymidine incorporation and production of several cytokines following incubation with four different mitogens. Results indicated that compared to barley-fed piglets, those fed wheat, Candle, and B-glc-fed had a significant increase in the stimulation index of PBL following incubation with LPS. However, the physiological importance of this is questioned due to the low level of incorporation of ^{3}H-thymidine in cells incubated with this mitogen. It may also be due to the slightly higher incorporation of ^{3}H-thymidine in the absence of mitogen by PBL of barley-fed piglets. Increasing the B-glc content of the diet increased ^{3}H-thymidine incorporation in MLN cells until the dietary concentration reached 35 g/kg. Feeding a high level of B-glc appeared to negatively impact the ability of these cells to incorporate ^{3}H-thymidine. Therefore, the mechanism by which increasing levels of B-glc influence MLN lymphocytes ex vivo is uncertain and requires further study. In addition, inclusion of the highest level of B-glc appears to somehow pre-condition PP lymphocyte response to mitogen (PHA & PWM), which was shown by the inverse relationship between increasing dietary B-glc content and decreasing ^{3}H-thymidine incorporation. There was also a significantly higher production of IL-10 by PBL of wheat-fed piglets in response to stimulation with PWM. Furthermore, production of this cytokine decreased with increasing B-glc until the dietary concentration reached 35 g/kg. Feeding the highest amount of B-glc appeared to positively influence production of this cytokine, which was similar to IL-10 production in piglets fed low levels of B-glc. Although anti-inflammatory properties are suggested, differences were small in magnitude and future studies are warranted to study the implications of these findings.

Gut Response to ETEC

In order for piglets to become infected with K88+ ETEC and display the clinical manifestations of diarrhea (Sarmiento et al. 1988), the bacteria must first adhere to the small intestine. This is accomplished by means of proteinaceous, filamentous appendages on the bacterial surface, called pili or fimbriae (Thorns et al. 1989). The K88 antigen is contained on this fimbrial structure and exists in at least three different antigenic forms with different adhesive specificities for brush border membrane components (Laux et al. 1986). In this study, FITC-labeled ETEC bound very rapidly to freshly isolated mucosal cells and absolute binding was close to 100% within 1 min. However the rate of intensity of binding (number of ETEC/cell) displayed a more gradual increase over time, which was influenced by diet (Figure 8). The higher rate of binding of ETEC to mucosal cells from barley-fed piglets suggests that diet can influence the ability of mucosal cells to bind ETEC. This effect appeared to be related to some attribute of the barley diet other than the B-glc content, since binding intensity was not directly related to the B-glc content of the diet. The physiological significance of these findings is difficult to determine. A major component that differed between these diets was total fiber (198.8 g/kg barley vs 141.4 g/kg Candle), especially Neutral Detergent Fiber (118.1 g/kg barley vs 42.81 g/kg Candle). K88 ETEC adhesins bind to glycoproteins and glycolipids (Jin & Zhao 2000) of various sizes in both intestinal mucus and brush border membranes (Grange & Mouricourt 1996). Therefore, it is possible that the fiber content of the diet influenced expression of glycoproteins and/or glycolipids in isolated mucosal cells. Alternatively, the abrasive properties of fiber, which have been shown to alter intestinal mucus composition (Tungland & Meyer 2002) may have influenced the proportion of glycoproteins and/or glycolipids in the overlying mucus layer. However, this was not likely the case as the mucosal cell isolation methods were designed to remove mucus.

Summary and Conclusion

Feeding diets with varying B-glc content (6.4 g/kg -73.7 g/kg) do not alter the growth rate of young piglets, nor do they influence the ability of systemic neutrophils to produce free radicals. The failure to see *in vitro* changes in neutrophil function may have resulted from an inability of B-glc to directly interact with immune cells *in vivo*. The beneficial effects of B-glc that have been reported *in vitro* may differ from *in vivo* studies do to metabolism of B-glc in the colon and thus not entering the circulation. Although feeding B-glc was shown to increase ileal ManP and G, changes in surface area would need to be considered before one could confidently conclude that feeding B-glc influences paracellular permeability. The B-glc content of the diet did not directly relate to the rate of *E. coli* binding to isolated enterocytes, but the higher rate of binding when additional B-glc were added to the Candle diet requires future study in an *in vivo* model. The finding that piglets fed the highest level of B-glc had an increase in blood lymphocytes, specifically antigen-naïve T cells, suggests that these animals may have an increased number of newly synthesized cells and perhaps a greater capacity to respond to immune challenge. However, this requires future study and the mechanisms remain unknown. In conclusion, feeding 74 g/kg B-glc by supplementing B-glc to the Candle diet decreased the proportion of peripheral T cells (CD2+), the proportion of naïve T cells (CD4+CD45RA+) in MLN, and T cell populations in PP (CD8-CD25+, CD4+CD45RA-, CD4+CD45RA+, total CD25+ and CD4+). However, supplementation of B-glc appeared to positively influence MLN B cells by increasing the proportion of IgG+ and CDw75+ cells. In addition, the decreased incorporation of ^{3}H-thymidine in unstimulated MLN cells and the increased production of IL-10 by PBL in response to PWM stimulation suggests potential benefits of feeding piglets a diet supplemented with B-glc.

TABLE 1 Composition of Experimental diets

Ingredient (g/kg)	1 – Wheat	2 - Barley	3 – Candle	4 – B-glc
Grain	537	512	485	485
SOYBEAN MEAL (48%)	150	150	150	150
CASEIN	106	131	158	158
FAT	100	100	100	100
TALLOW	*55.56*	*55.56*	*55.56*	*55.56*
SUNFLOWER OIL	*37.21*	*37.21*	*37.21*	*37.21*
LINSEED OIL	*7.23*	*7.23*	*7.23*	*7.23*
LIMESTONE	6.00	6.00	6.00	6.00
DICALCIUM PHOSPHATE (21%)	24.0	24.0	24.0	24.0
SALT	2.50	2.50	2.50	2.50
SWINE PC G/F VITAMIN PREMIX[1]	1.00	1.00	1.00	1.00
PC SWINE TRACE MINERAL PREMIX[2]	2.00	2.00	2.00	2.00
CYSTEINE	1.20	1.20	1.20	1.20
VITAMIN B-12	$1.67 \text{ X } 10^{-5}$	$1.67 \text{ X } 10^{-5}$	$1.67 \text{ X } 10^{-5}$	$1.67 \text{ X } 10^{-5}$
SUPPLEMENT	70.0	70.0	70.0	70.0
CASEIN	*4.82*	*4.82*	*4.82*	
CANDLE CRUDE STARCH	*12.4*	*12.4*	*12.4*	
NON-NUTRITIVE FIBER	*52.8*	*52.8*	*52.8*	
VISCOFIBER™				*70.0*
Total	**1000**	**1000**	**1000**	**1000**

[1] The vitamin premix contained: 3.8912% Protein, 0.99% Fat, 2.701% Moisture, 86.002 kcal/kg DE, 82.0002 kcal/kg ME, 25.7937% Calcium, 0.225% Phosphorus, 0.0765% Av Phosphorus, 0.1282% Magnesium, 0.0328% Sodium, 642.2313 mg/kg iron, 7000014.2 IU/kg Vitamin A, 700014.2 IU/kg Vitamin D3, 20000.0117 IU/kg Vitamin E, 1500.0179 mg/kg Vitamin K, 40.0001 mg/kg Biotin, 399.9008 mg/kg Folic Acid, 20000.0117 mg/kg Niacin, 7499.9741 mg/kg Pantothenic Acid, 533.9845 mg/kg pyridoxine, 3000.0051 mg/kg riboflavin, 580.5812 mg/kg thiamine, 10 mg/kg Vitamin B12

[2] The trace mineral premix contained: 0.99% fat, 0.001% moisture, 86 kcal/kg DE, 82 kcal/kg ME, 15.1874% Calcium, 0.0729% Magnesium, 0.0283% Sodium, 4.756% Sulfur, 351 mg/kg cobalt, 5000 mg/kg Copper, 749.3 mg/kg Iodine, 75365.1328 mg/kg Iron, 25020 mg/kg Manganese, 150 mg/kg Selenium, 75024 mg/kg Zinc, 100.001 g/kg Choline

TABLE 2 Chemical composition of B-glc experimental diets calculated from proximate analysis of individual dietary ingredients given in Appendix (Table 1).

	1 - Wheat	**2 - Barley**	**3 – Candle**	**4 – Candle + B-glc supplement**
DE kcal/kg	3702	3560	3740	3740
ME kcal/kg	3510	3364	3580	3580
Crude Protein (g/kg)	260.3	260.7	260.7	260.7
Crude Fat (g/kg)	121.1	119.3	118.5	118.5
ADF (g/kg)	17.01	27.93	45.97	84.52
NDF (g/kg)	98.19	118.1	42.81	54.93
Total fiber (g/kg)	168.0	198.8	141.5	139.4
Total B-glc (g/kg)	6.416	17.25	35.53	73.69

TABLE 3. BW, FI & intestinal indices of piglets fed diets differing in B-glc content

Group	Wheat (n=7)	Barley (n=8)	Candle (n=8)	Bglucan (n=8)
Initial BW^1,*g*	$5980^2 \pm 424$	6226 ± 130	6435 ± 270	6513 ± 241
Final BW, *g*	7421 ± 138	7470 ± 144	7414 ± 196	7350 ± 259
ADG^3, g	1441 ± 314	1245 ± 141	979 ± 295	837 ± 401
FI^4, *g/d*	$252^{b5} \pm 11$	$177^a \pm 20$	$158^a \pm 24$	$166^a \pm 26$
FI, *g/kg BW*	$37^b \pm 1$	$25^a \pm 3$	$23^a \pm 3$	$23^a \pm 4$
Intestinal Weight, *g*	380.4 ± 18	319.3 ± 20	283.8 ± 22	320.6 ± 39
Intestinal Length, *cm*	1031 ± 27	939.8 ± 16	973.1 ± 48	999.9 ± 55

[1] Body Weight

[2] Values presented as mean ± SEM

[3] Average Daily Gain

[4] Feed Intake

[5] Means within a row that do not share a common letter are significantly different ($p<0.05$)

TABLE 4. Blood chemistry measures of piglets at 35 days of age and fed diets differing in B-glc

Group	Normal Range	Wheat (n=7)	Barley (n=8)	Candle (n=8)	Bglucan (n=8)
HGB[1]	100-160 g/L[2]	120[3] ± 2	117 ± 4	123 ± 3	127 ± 4
RBC[1]	5.0-8.0 x10^{12}/L	6^{a}[4] ± 0.08	6^{a} ± 0.2	7^{ab} ± 0.08	7^{b} ± 0.2
MCV[1]	50-68 fl	59 ± 1	57 ± 0.9	57 ± 1	56 ± 1
MCH[1]	17-21 pg	19 ± 0.2	18 ± 0.3	18 ± 0.4	18 ± 0.3
MCHC[1]	300-340 g/L	318 ± 1	322 ± 1	320 ± 2	321 ± 2
RDW[1]	n.a. (%)	17 ± 1	20 ± 1	20 ± 2	19 ± 1
HCT[1]	n.a. (L/L)	0.4 ± 0.005	0.4 ± 0.01	0.4 ± 0.01	0.4 ± 0.01
PLT[1]	n.a. (10^{9}/L)	454 ± 56	387 ± 22	482 ± 48	371 ± 47
MPV[1]	n.a. (fL)	8 ± 0.3	8 ± 0.2	8 ± 0.3	8 ± 0.3
WBC[1]	11.0-22.0 x 10^{9}/L	12^{a} ± 1	16^{b} ± 1	13^{a} ± 1	17^{b} ± 2
Lymphocyte	4.2-13.6 x 10^{9}/L	8^{a} ± 1	11^{ab} ± 1	9^{a} ± 1	13^{b} ± 2
Neutrophil	3.0-10.0 x 10^{9}/L	4 ± 1	5 ± 1	3 ± 0.5	4 ± 1
Monocyte	0.2-2.2 x 10^{9}/L	0.3 ± 0.04	0.5 ± 0.1	0.3 ± 0.04	0.5 ± 0.2
Eosinophil	0.5-2.4 x 10^{9}/L	0.06^{a} ± 0.01	0.1^{b} ± 0.02	0.05^{a} ± 0.005	0.07^{ab} ± 0.01
Basophil	0-0.4 x 10^{9}/L	0.07 ± 0.01	0.1 ± 0.02	0.1 ± 0.05	0.2 ± 0.1
Lymphocytes	n.a. (%)	68 ± 5	67 ± 3	69 ± 4	73 ± 3
Neutrophils	n.a. (%)	29 ± 5	28 ± 3	27 ± 3	23 ± 4
Monocytes	n.a. (%)	2 ± 0.2	3 ± 0.7	3 ± 0.4	3 ± 0.4
Eosinophils	n.a. (%)	0.5 ± 0.1	0.6 ± 0.08	0.4 ± 0.06	0.5 ± 0.1
Basophils	n.a. (%)	0.5 ± 0.04	0.8 ± 0.1	1 ± 0.5	0.9 ± 0.3

[1] HGB-Hemoglobin, RBC-Red Blood Cells, MCV-Mean Corpuscular Volume, MCH-Mean Corpuscular Hemoglobin, MCHC-Mean Corpuscular Hemoglobin Concentration, RDW-Red Cell Distribution Width, HCT-Hematocrit, PLT-Platelets, MPV-Mean Platelet Volume, WBC – White Blood Cells

[2] Ranges obtained from (Blood & Rodostits 1989), values that were not given are indicated with n.a.

[3] Values presented as mean ± SEM

[4] Means within a row that do not share a common letter are significantly different ($p<0.05$)

TABLE 5. Immune Cell Populations Blood Concentrations of piglets fed diets differing in B-glucan content

Group	Wheat (n=7)	Barley (n=8)	Candle (n=8)	Bglucan (n=8)
IgA	1.1[1] ± 0.4	0.8 ± 0.2	0.7 ± 0.1	1.4 ± 0.6
IgM	1.7 ± 0.5	2.0 ± 0.4	1.5 ± 0.3	2.8 ± 1.1
IgG	1.5 ± 0.2	2.0 ± 0.3	1.5 ± 0.4	2.3 ± 0.5
B cell	1.2 ± 0.2	1.8 ± 0.3	1.2 ± 0.3	2.0 ± 0.5
CD3	0.1 ± 0.1	0.1 ± 0.1	0.2 ± 0.1	0.2 ± 0.2
CD45RA	3.9^{a}[2] ± 0.6	5.4^{ab} ± 0.7	4.2^{a} ± 0.7	6.9^{b} ± 1.3
CD4	1.4^{a} ± 0.1	1.6^{a} ± 0.2	1.7^{a} ± 0.3	2.3^{b} ± 0.3
CD8	1.0 ± 0.1	1.5 ± 0.3	1.4 ± 0.3	2.3 ± 0.7

[1] Values presented as mean concentration (10^9/L) ± SEM

[2] Means within a row that do not share a common letter are significantly different ($p<0.05$)

TABLE 6. Neutrophil Burst, Size and Granularity measures of piglets (35 days of age)

Group	Wheat (n=7)	Barley (n=8)	Candle (n=8)	Bglucan (n=8)
Burst				
0 min.	4^1 ± 1	12 ± 9	6 ± 2	5 ± 1
5 min.	51 ± 14	61 ± 12	68 ± 15	54 ± 15
Fold inc. (0-5 min.)	16 ± 4	15 ± 4	15 ± 3	12 ± 3
10 min.	183 ± 36	245 ± 29	222 ± 38	193 ± 38
Fold inc. (0-10 min.)	62 ± 12	65 ± 13	55 ± 10	51 ± 9
Fold inc. (5-10 min.)	5 ± 1	5 ± 1	4 ± 0.5	5 ± 1
15 min.	303 ± 47	386 ± 41	341 ± 51	322 ± 48
Fold inc. (0-15 min.)	111 ± 22	100 ± 20	86 ± 18	90 ± 20
Fold inc. (10-15 min.)	2 ± 0.3	2 ± 0.1	2 ± 0.1	2 ± 0.1
Size				
0 min.	441 ± 9	436 ± 14	425 ± 9	419 ± 10
5 min.	463 ± 16	467 ± 12	466 ± 11	450 ± 10
10 min.	474 ± 16	481 ± 10	473 ± 12	468 ± 9
15 min.	487 ± 13	475 ± 12	487 ± 11	479 ± 8
Granularity				
0 min.	290 ± 28	314 ± 21	299 ± 26	302 ± 23
5 min.	272 ± 17	278 ± 15	278 ± 16	276 ± 17
10 min.	271 ± 20	280 ± 18	271 ± 17	278 ± 20
15 min.	281 ± 24	272 ± 19	282 ± 24	287 ± 25

[1] Values presented as mean fluorescence ± SEM, there were no significant differences among dietary treatments ($P<0.05$)

Table 7. Lymphocyte Distribution in PBL of piglets

Antibody	Wheat (n=7)	Barley (n=8)	Candle (n=8)	Bglucan (n=8)
IgA	13[1] ± 5	8 ± 1	9 ± 2	9 ± 3
IgM	20 ± 6	18 ± 3	16 ± 2	20 ± 4
IgG	19 ± 2	18 ± 3	17 ± 3	18 ± 3
CDw75	14 ± 2	10 ± 2	15 ± 1	12 ± 1
CD11b	1 ± 0.4	1 ± 0.4	1 ± 0.4	2 ± 1
CD4+CD25+	3 ± 0.5	2 ± 0.4	3 ± 1	4 ± 1
CD8+CD25+	1 ± 0.2	1 ± 0.4	1 ± 0.3	2 ± 1
CD4+CD45RA+	6 ± 1	7 ± 2	7 ± 1	7 ± 1
CD4+CD45RA-	13 ± 1	9 ± 1	12 ± 1	11 ± 1
% CD4 RA+	31 ± 3	36 ± 5	35 ± 3	36 ± 4
CD8+CD45RA+	5 ± 1	5 ± 1	8 ± 3	10 ± 3
CD8+CD45RA-	8 ± 1	7 ± 1	9 ± 1	8 ± 2
% CD8 RA+	39 ± 3^{a}[2]	44 ± 5^{ab}	42 ± 4^{a}	49 ± 5^{b}
CD4+	17 ± 1	15 ± 2	19 ± 2	18 ± 1
CD8+	12 ± 1	13 ± 2	15 ± 2	17 ± 4
CD4/CD8 ratio	1 ± 0.1	1 ± 0.2	1 ± 0.2	2 ± 0.4
RA+	46 ± 6	50 ± 5	47 ± 5	53 ± 4
CD25+	13 ± 2	13 ± 2	14 ± 1	14 ± 2
CD44+	91 ± 5	92 ± 2	95 ± 1	90 ± 5
Monocyte+	9 ± 2	9 ± 2	8 ± 2	7 ± 2
B cell+	14 ± 1	16 ± 2	13 ± 3	15 ± 2
CD2+	30 ± 4	40 ± 5	49 ± 5	42 ± 4

[1] Values presented as cell population percentage ± SEM

[2] Means within a row that do not share a common letter are significantly different ($p<0.05$)

Table 8. Cell Phenotyping from MLN Lymphocytes

Antibody	Wheat (n=7)	Barley (n=8)	Candle (n=8)	Bglucan (n=8)
IgA	22[1] ± 2	18 ± 2	17 ± 3	22 ± 3
IgM	41 ± 3	38 ± 3	37 ± 3	41 ± 5
IgG	42 ± 3	36 ± 3	33 ± 3	40 ± 4
CDw75	32 ± 2	24 ± 2	23 ± 3	28 ± 4
CD11b	4 ± 1	3 ± 1	3 ± 1	4 ± 1
CD4+CD25+	6 ± 1	6 ± 0.4	7 ± 1	6 ± 1
CD8+CD25+	2 ± 0.2	2 ± 0.3	3 ± 0.3	2 ± 0.3
CD4+CD45RA+	6^{a}[2] ± 1	10^{b} ± 1	10^{b} ± 1	8^{ab} ± 1
CD4+CD45RA-	24 ± 3	22 ± 2	26 ± 2	23 ± 2
% CD4 RA+	21 ± 3^{a}	31 ± 3^{b}	27 ± 2^{ab}	27 ± 3^{ab}
CD8+CD45RA+	9 ± 1	11 ± 1	9 ± 1	10 ± 1
CD8+CD45RA-	10 ± 1	10 ± 1	12 ± 1	10 ± 1
% CD8 RA+	47 ± 2	51 ± 2	43 ± 4	51 ± 2
CD4+	31 ± 3	32 ± 2	35 ± 2	31 ± 3
CD8+	17 ± 2	20 ± 1	19 ± 2	19 ± 1
CD4/CD8 ratio	2 ± 0.2	2 ± 0.1	2 ± 0.1	2 ± 1
RA+	61 ± 4	62 ± 4	57 ± 4	62 ± 4
CD25+	29 ± 4	26 ± 2	25 ± 3	25 ± 2
CD44+	85 ± 4	84 ± 3	83 ± 3	80 ± 3
Monocyte+	17 ± 3	16 ± 2	18 ± 2	18 ± 2
B cell+	35 ± 3	36 ± 2	31 ± 3	36 ± 3
CD2+	35 ± 7	32 ± 5	30 ± 4	29 ± 4

[1] Values presented as cell population percentage ± SEM

[2] Means within a row that do not share a common letter are significantly different ($p<0.05$)

TABLE 9. Cell Phenotyping from PP of piglets

Antibody	Wheat (n=7)	Barley (n=8)	Candle (n=8)	Bglucan (n=8)
IgA	11^{1} ± 3	14 ± 3	16 ± 4	13 ± 3
IgM	59 ± 6	57 ± 5	56 ± 5	58 ± 4
IgG	45 ± 5	45 ± 5	50 ± 4	48 ± 4
CDw75	40 ± 4	41 ± 4	47 ± 4	46 ± 3
CD11b	1 ± 0.1	1 ± 0.1	1 ± 0.4	1 ± 0.2
PP Bcell	5 ± 2	7 ± 1	8 ± 3	5 ± 1
CD4+CD25+	1 ± 0.1	1 ± 0.2	1 ± 0.3	1 ± 0.3
CD8+CD25+	1 ± 0.2	1 ± 0.1	2 ± 1	1 ± 0.3
CD4+CD45RA+	0.2^{a2} ± 0.04	0.2^{a} ± 0.04	0.4^{b} ± 0.1	0.3^{a} ± 0.06
CD4+CD45RA-	1 ± 0.1	2 ± 0.4	2 ± 1	1 ± 0.3
% CD4 RA+	13 ± 2	15 ± 2	16 ± 3	18 ± 3
CD8+CD45RA+	1 ± 0.1	1 ± 0.2	1 ± 0.4	1 ± 0.2
CD8+CD45RA-	2 ± 0.3	3 ± 0.5	3 ± 1	2 ± 1
% CD8 RA+	23 ± 1	29 ± 2	29 ± 2	29 ± 3
CD4+	2 ± 0.2	2 ± 0.4	3 ± 1	2 ± 0.4
CD8+	3 ± 0.3	4 ± 1	5 ± 2	3 ± 1
CD4/CD8 ratio	1 ± 0.04	1 ± 0.1	1 ± 0.1	1 ± 0.1
RA+	89 ± 2	84 ± 3	89 ± 3	87 ± 3
CD25+	6 ± 1	8 ± 1	11 ± 3	6 ± 1
CD44+	15 ± 2	16 ± 2	21 ± 5	17 ± 2
Monocyte+	6 ± 1	7 ± 1	10 ± 3	9 ± 2
B cell+	30 ± 5	31 ± 4	37 ± 3	33 ± 4

[1] Values presented as cell population percentage ± SEM

[2] Means within a row that do not share a common letter are significantly different ($p<0.05$)

Table 10. ^{3}**H-Thymidine Uptake by PBL, MLN and PP of piglets fed diets varying in B-glc content**

Tissue (mitogen) -	Measure	Wheat (n=7)	Barley (n=8)	Candle (n=8)	Bglucan (n=8)
PBL (none)	DPM[1]	1454 ± 354	2033 ± 475	1423 ± 347	1306 ± 181
PBL (PHA)	DPM	161240 ± 45367	208257 ± 24511	253114 ± 41395	236373 ± 19114
	SI[2]	2.0 ± 0.2	2.0 ± 0.1	2.3 ± 0.1	2.3 ± 0.1
PBL (PMAI)	DPM	129715 ± 31478	158263 ± 15882	119090 ± 22008	138534 ± 29196
Log transformed[3]	SI	1.9 ± 0.2	1.9 ± 0.1	1.9 ± 0.2	1.9 ± 0.1
PBL (PWM)	DPM	140518 ± 32055	155149 ± 19865	194464 ± 27620	166287 ± 14630
Log transformed	SI	2.0 ± 0.2	1.9 ± 0.1	2.2 ± 0.1	2.1 ± 0.1
PBL (LPS)	DPM	6030 ± 1676	4074 ± 1013	4454 ± 971	5095 ± 1204
Log transformed	SI	0.5^{b4} ± 0.1	0.2^{a} ± 0.2	0.5^{b} ± 0.1	0.5^{b} ± 0.1
MLN (none) Log transformed	DPM	2.5 ± 0.05	2.5 ± 0.1	2.8 ± 0.1	2.6 ± 0.1
MLN (PHA)	DPM	196343 ± 21323	208262 ± 24959	195872 ± 16541	174720 ± 29988
	SI	663 ± 93	748 ± 194	370 ± 106	420 ± 91
MLN (PMAI)	DPM	159118 ± 11362	194803 ± 19829	167498 ± 9661	160746 ± 18106
Log transformed	SI	2.7 ± 0.03	2.8 ± 0.1	2.4 ± 0.1	2.5 ± 0.1
MLN (PWM)	DPM	170746 ± 17724	160081 ± 27505	190357 ± 12261	187444 ± 25264
	SI	573 ± 93	564 ± 155	374 ± 120	447 ± 84
MLN (LPS)	DPM	1131 ± 494	1048 ± 410	2407 ± 1081	1351 ± 501
Log transformed	SI	0.3 ± 0.2	0.3 ± 0.1	0.2 ± 0.2	0.3 ± 0.2
PP (none) Log transformed	DPM	2.4 ± 0.1	2.4 ± 0.2	2.4 ± 0.1	2.3 ± 0.1
PP (PHA) Log transformed	DPM	3.2 ± 0.2	3.2 ± 0.2	2.9 ± 0.1	2.8 ± 0.1
Log transformed	SI	0.8 ± 0.2	0.8 ± 0.2	0.5 ± 0.1	0.5 ± 0.1
PP (PMAI)	DPM	79892 ± 31929	67142 ± 28048	67798 ± 28026	40041 ± 12460
Log transformed	SI	2.2 ± 0.2	2.1 ± 0.2	2.2 ± 0.2	2.0 ± 0.2
PP (PWM) Log transformed	DPM	3.1 ± 0.2	2.9 ± 0.2	2.8 ± 0.1	2.6 ± 0.1
Log transformed	SI	0.7 ± 0.2	0.5 ± 0.3	0.4 ± 0.1	0.3 ± 0.1
PP (LPS) Log transformed	DPM	2.7 ± 0.2	2.7 ± 0.2	2.7 ± 0.2	2.4 ± 0.1
Log transformed	SI	0.3 ± 0.1	0.3 ± 0.2	0.3 ± 0.1	0.1 ± 0.1

[1] Reported in Decays per Minute ± SEM

[2] Reported as mean Stimulation Index (calculated as DPM stimulated/DPM unstimulated)

[3] Kurtosis and Skew were analyzed and values that were not considered normally distributed were log transformed.

[4] Means within a row that do not share a common letter are significantly different ($p<0.05$)

TABLE 11. Cytokine Production by PBL in piglets fed diets of differing B-glc content

Cytokine	***Mitogen***	**Wheat (n=7)**	**Barley (n=8)**	**Candle (n=8)**	**Bglucan (n=8)**
IL-4	PMAI	36 ± 8[1]	21 ± 4	26 ± 6	20 ± 6
Log transformed	PHA	1.5 ± 0.1	1.8 ± 0.1	1.7 ± 0.1	1.7 ± 0.1
IL-2	PMAI	9063 ± 1613	10097 ± 1117	7281 ± 796	8274 ± 1656
	PHA	1238 ± 443	1508 ± 268	1639 ± 282	1574 ± 338
IL-10 (Log transformed)[2]	PMAI	1.2 ± 0.1	1.2 ± 0.03	1.3 ± 0.1	1.3 ± 0.1
Log transformed	PHA	1.1 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.05
Log transformed	PWM	1.5 ± 0.1	1.3 ± 0.04	1.3 ± 0.1	1.5 ± 0.1
Log transformed	LPS	1.4 ± 0.1	1.4 ± 0.1	1.3 ± 0.1	1.2 ± 0.1
IFN-γ	PMAI	2728 ± 875	3782 ± 1017	3105 ± 1349	3355 ± 1000
	PHA	444 ± 193	1254 ± 455	1105 ± 502	971 ± 444
	PWM	517 ± 279	982 ± 304	882 ± 247	405 ± 95
TNF-α	PHA	21 ± 6	28 ± 7	28 ± 6	30 ± 7

[1] Values presented as mean ± SEM, there were no significant differences among dietary treatments ($P<0.05$)

[2] Kurtosis and Skew were analyzed for cytokine data and values that were not considered normally distributed were log transformed.

TABLE 12. Cytokine Production by MLN in piglets fed diets differing in B-glc content

Cytokine	***Mitogen***	**Wheat (n=7)**	**Barley (n=8)**	**Candle (n=8)**	**Bglucan (n=8)**
IL-4	PMAI	16 ± 4[1]	16 ± 5	24 ± 9	19 ± 4
	PHA	114 ± 40	76 ± 18	78 ± 18	78 ± 14
IL-2	PMAI	8859 ± 1388	6661 ± 1098	8823 ± 1423	8649 ± 1638
	PHA	905 ± 222	1310 ± 458	1388 ± 381	1296 ± 310
IL-10	PHA	26 ± 7	18 ± 3	22 ± 5	24 ± 4
IFN-γ (Log transformed)[2]	PMAI	3.3 ± 0.1	3.3 ± 0.1	3.2 ± 0.1	3.2 ± 0.1
	PHA	666 ± 256	367 ± 90	407 ± 143	493 ± 171
Log transformed	PWM	1.8 ± 0.3	1.9 ± 0.3	2.1 ± 0.2	1.9 ± 0.2
TNF-α (Log transformed)	PHA	1.1 ± 0.1	1.1 ± 0.04	1.2 ± 0.1	1.2 ± 0.1

[1] Values presented as mean ± SEM, there were no significant differences among dietary treatments ($P<0.05$)

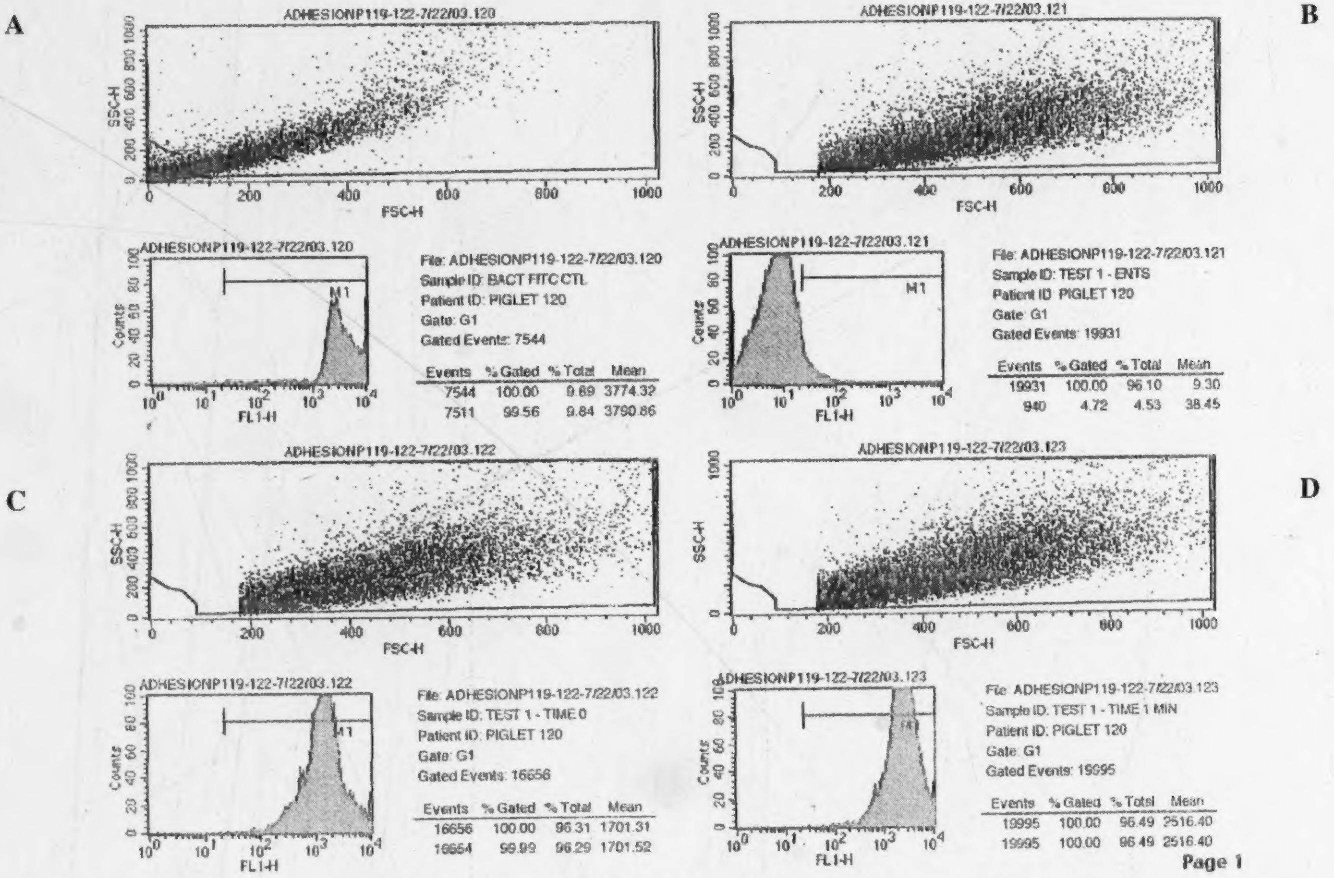
[3] Kurtosis and Skew were analyzed for cytokine data and values that were not considered normally distributed were log transformed.

Table 13. Permeability and Electrical Measures of intestinal loops in USSING chambers (35 days of age)

Dietary Group	Wheat (n=7)	Barley (n=8)	Candle (n=8)	B-glc (n=8)
ManP (nm/cm/hr)				
Jejunum	11.85[1] ± 2.3	13.55 ± 2.2	11.36 ± 1.4	10.85 ± 2.0
Ileum	7.72^{a}[2] ± 1.0	11.20^{ab} ± 1.6	7.92^{a} ± 1.7	16.64^{b} ± 4.0
G (A/cm^{2})				
Jejunum	19.20 ± 1.5	19.71 ± 3.8	24.16 ± 1.2	23.22 ± 1.6
Ileum	22.54^{a} ± 2.1	20.43^{a} ± 1.6	25.35^{a} ± 1.9	27.43^{b} ± 6.4
Isc (uA/cm2)				
Jejunum	12.50 ± 2.5	9.084 ± 1.3	17.60 ± 3.6	52.30 ± 25
Ileum	12.92 ± 3.5	21.13 ± 9.8	10.44 ± 5.1	13.21 ± 4.1
PD (mV)				
Jejunum	0.664 ± 0.14	0.375 ± 0.084	0.764 ± 0.18	1.836 ± 0.74
Ileum	0.540 ± 0.14	1.050 ± 0.48	0.395 ± 0.18	0.638 ± 0.23
Forskolin (Δ Isc)				
Jejunum	38.19 ± 8.6	39.47 ± 7.8	52.74 ± 11	43.50 ± 9.2
Ileum	26.99 ± 1.8	37.17 ± 17	45.32 ± 8.1	34.37 ± 15

[1] Values presented as mean ± SEM

[2] Significant comparisons at the 0.05 level are indicated with different letters



File: ADHESIONP119-122-7/22/03.120
Sample ID: BACT FITC CTL
Patient ID: PIGLET 120
Gate: G1
Gated Events: 7544

Events	% Gated	% Total	Mean
7544	100.00	9.89	3774.32
7511	99.56	9.84	3790.86

File: ADHESIONP119-122-7/22/03.121
Sample ID: TEST 1 - ENTS
Patient ID: PIGLET 120
Gate: G1
Gated Events: 19931

Events	% Gated	% Total	Mean
19931	100.00	96.10	9.30
940	4.72	4.53	38.45

File: ADHESIONP119-122-7/22/03.122
Sample ID: TEST 1 - TIME 0
Patient ID: PIGLET 120
Gate: G1
Gated Events: 16656

Events	% Gated	% Total	Mean
16656	100.00	96.31	1701.31
16654	99.99	96.29	1701.52

File: ADHESIONP119-122-7/22/03.123
Sample ID: TEST 1 - TIME 1 MIN
Patient ID: PIGLET 120
Gate: G1
Gated Events: 19995

Events	% Gated	% Total	Mean
19995	100.00	96.49	2516.40
19995	100.00	96.49	2516.40

Figure 1. Flow Cytometry Printout of Adhesion Assay. A) Analysis of ETEC-FITC control with no mucosal cells B) Analysis of Mucosal Cells only C) Analysis of mucosal cells immediately following addition of ETEC-FITC D) Analysis of mucosal cells 1 min. following addition of ETEC-FITC.

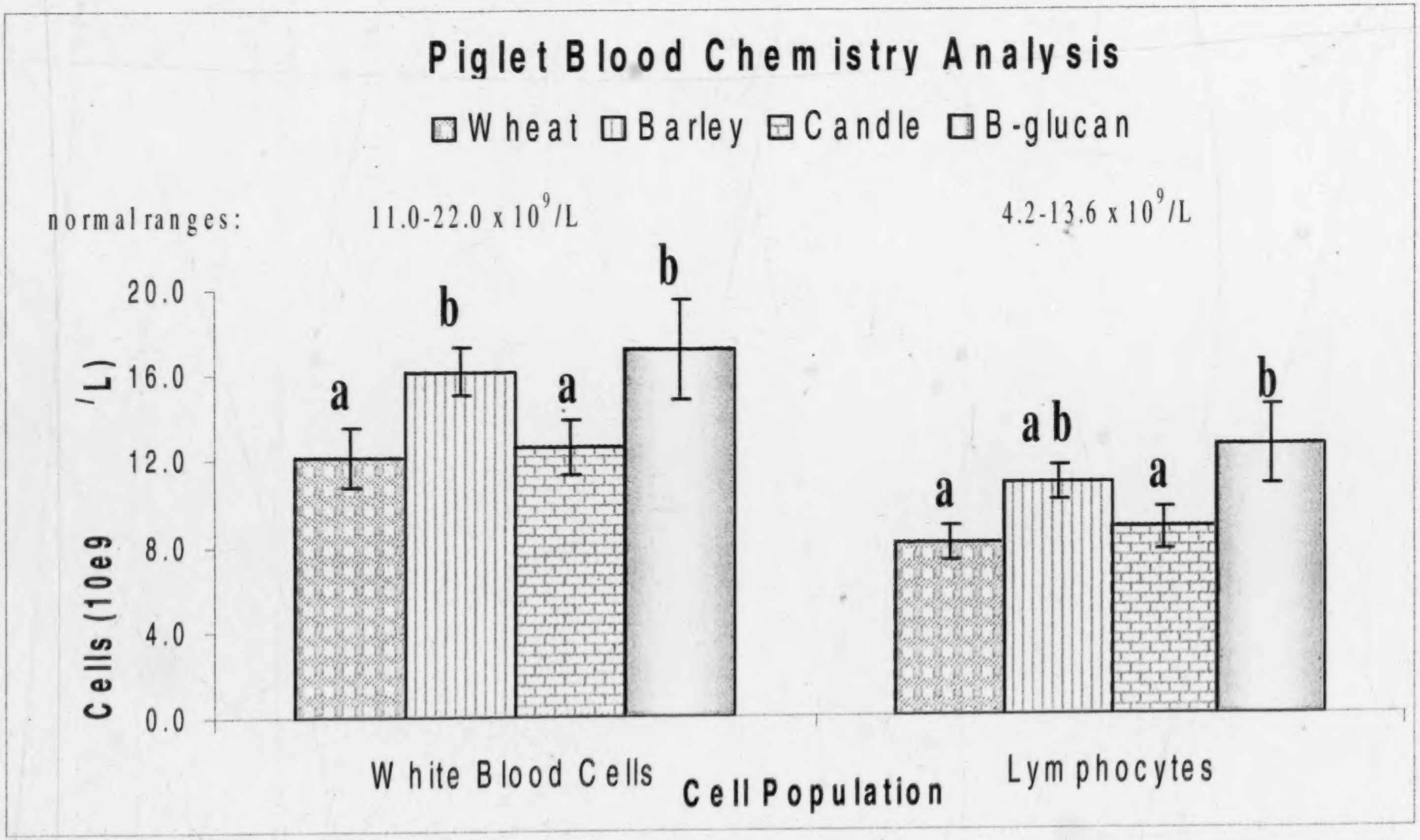


Figure 2. Blood chemistry concentrations of piglets (n=8) fed diets of increasing B-glc concentration. Normal ranges obtained from (Blood & Rodostits 1989). Indicated as mean cell concentration (10^9/L) with error bars representing SEM. Concentrations that do not share a common letter are significantly different ($p<0.05$).

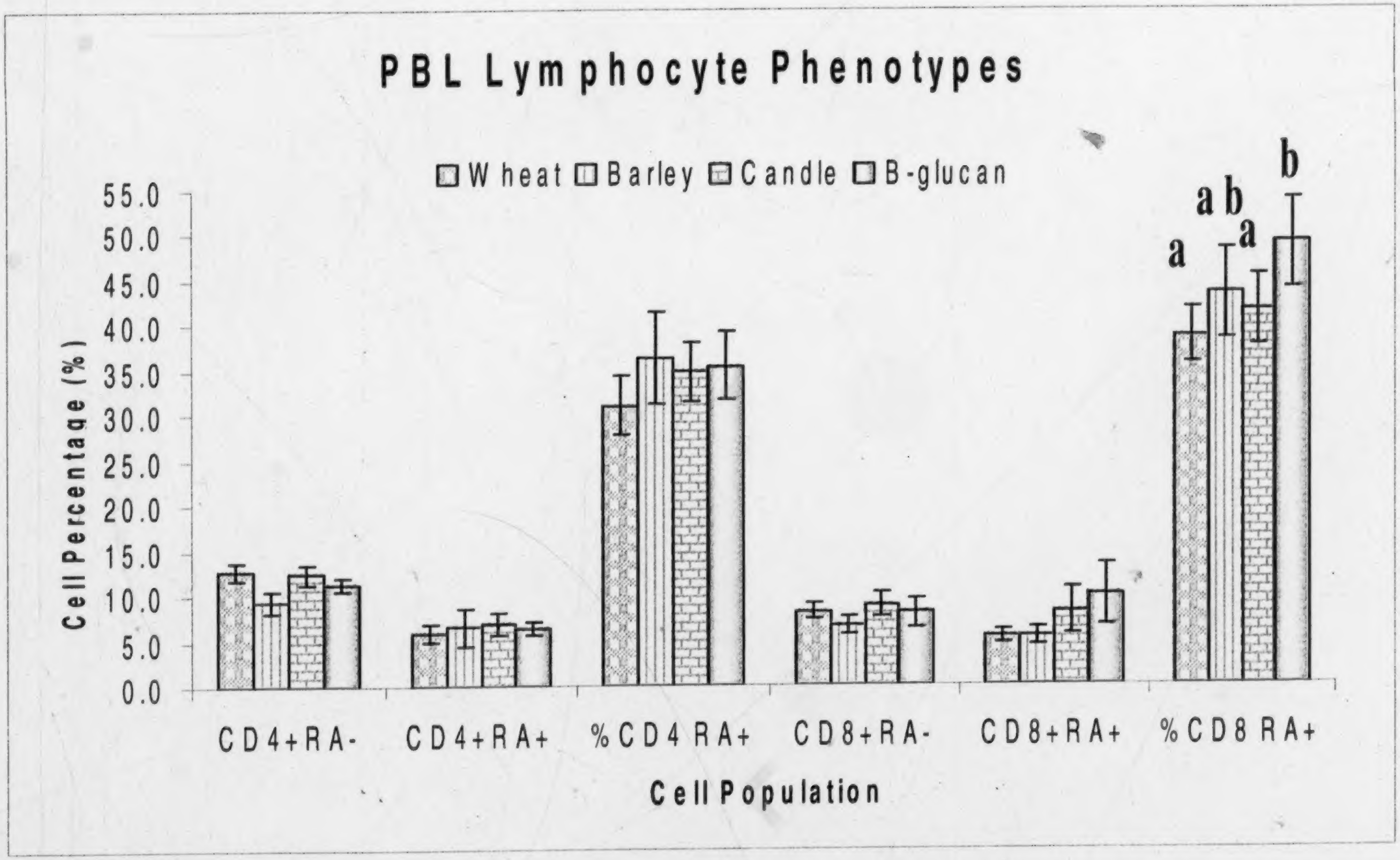


Figure 3. PBL immune cell phenotype expression of piglet CD45RA on T cells. Phenotypes given as mean with error bars representing SEM. Phenotypes that do not share a common letter are significantly different ($p<0.05$).

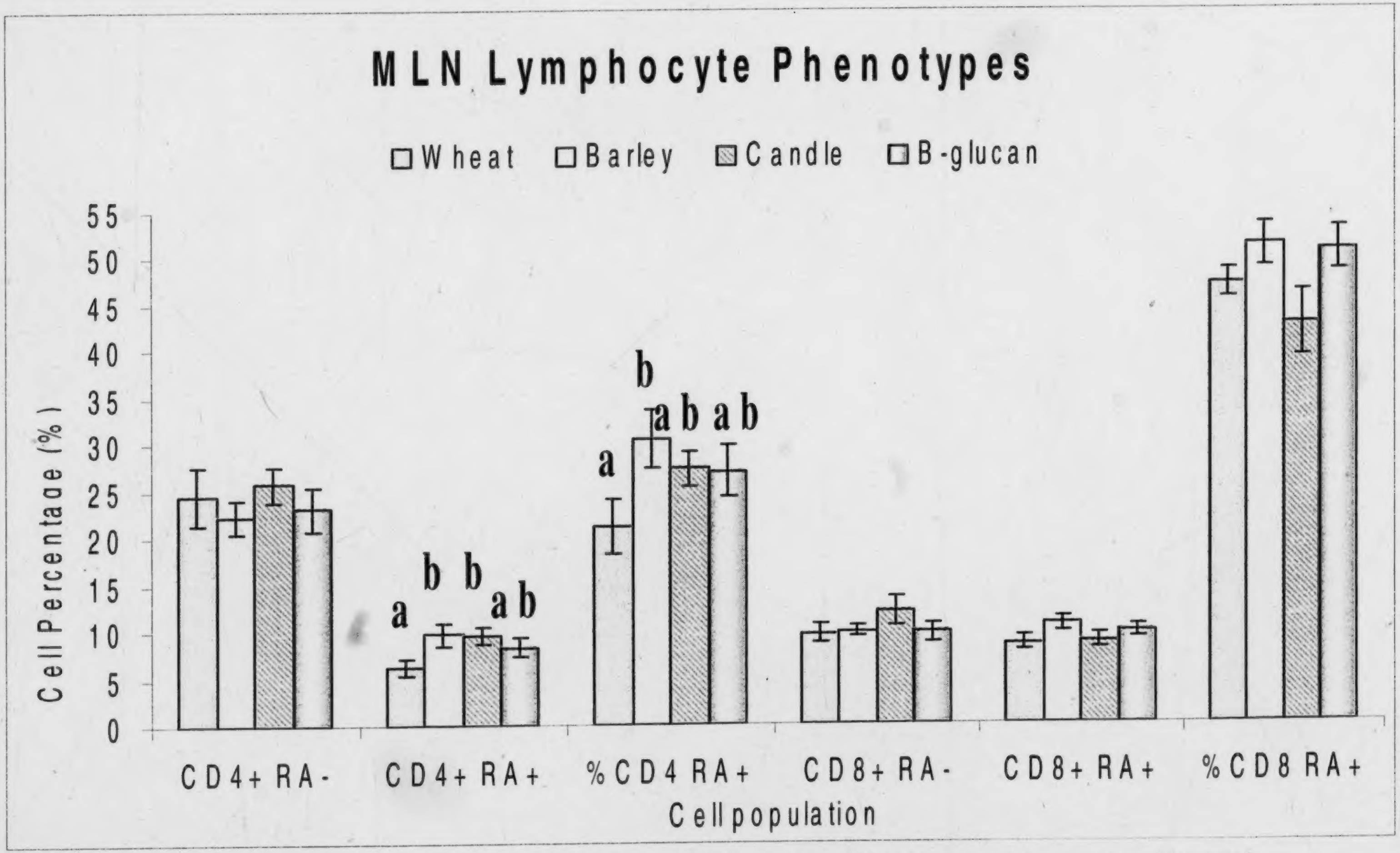


Figure 4. MLN Immune cell phenotype expression of piglet CD45RA on T cells. Phenotypes given as mean with error bars representing SEM. Phenotypes that do not share a common letter are significantly different ($p<0.05$).

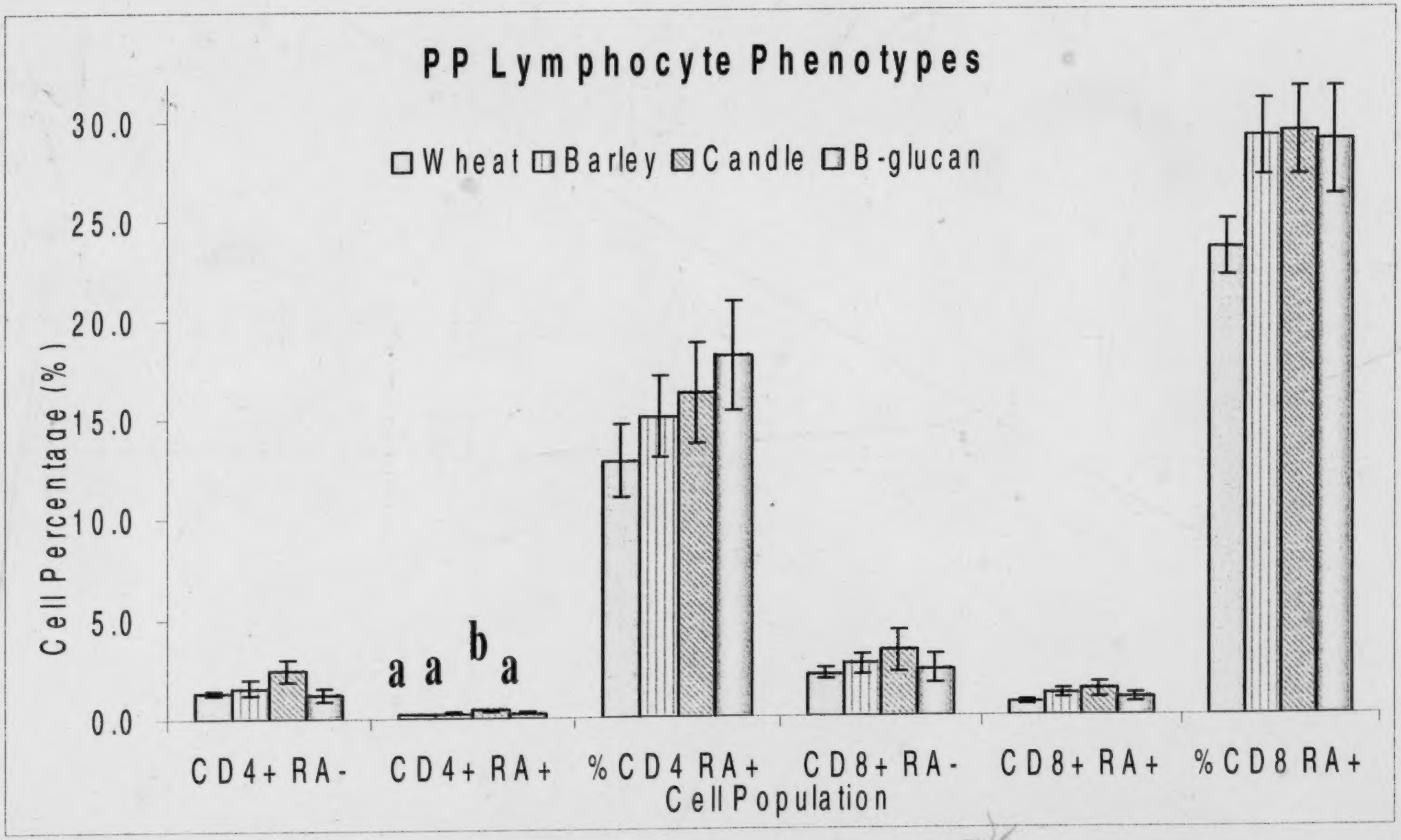


Figure 5. PP Immune cell phenotype expression of piglet CD45RA on T cells. Phenotypes given as mean with error bars representing SEM. Phenotypes that do not share a common letter are significantly different ($p<0.05$).

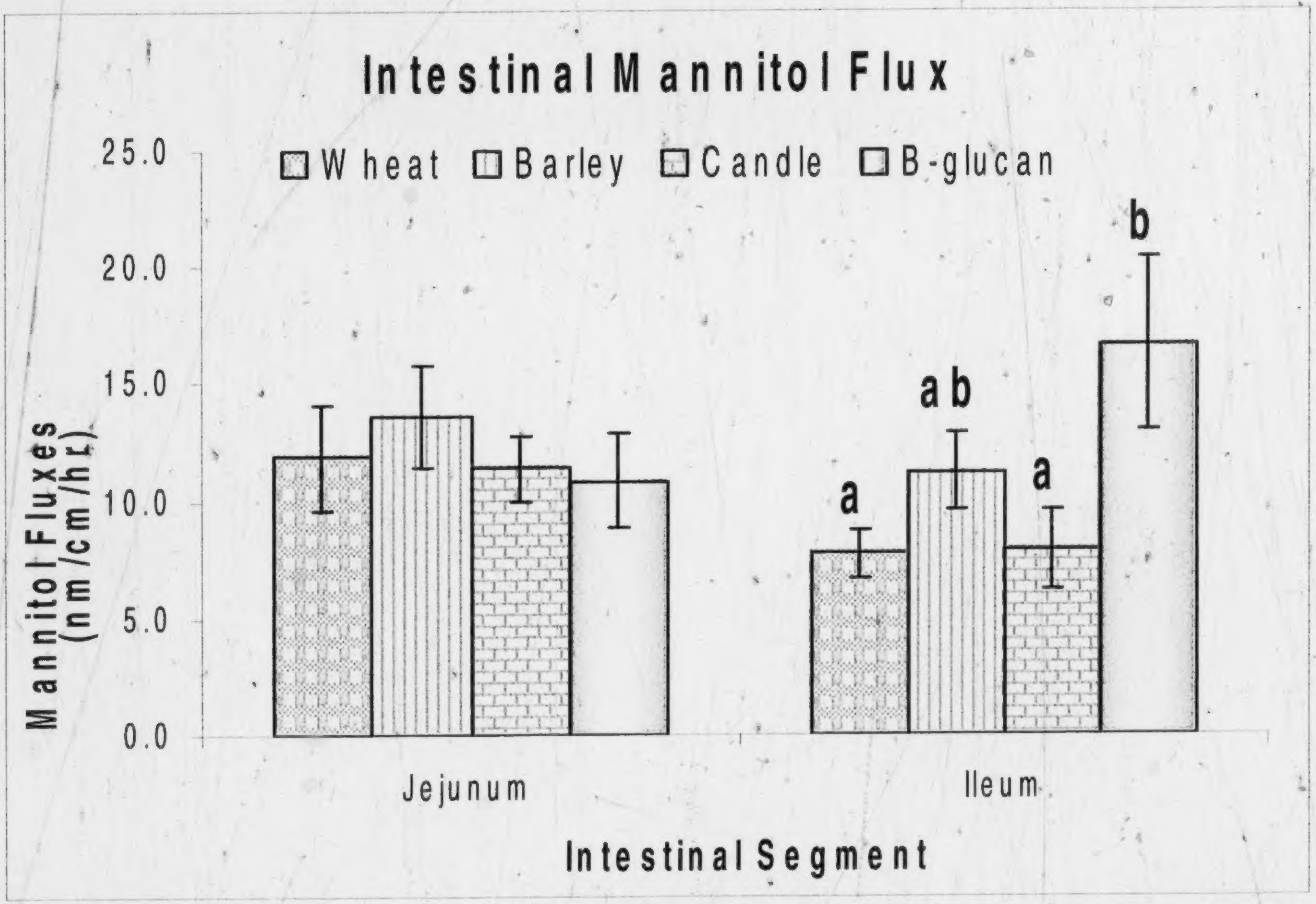


Figure 6. Intestinal ManP measured in Ussing Chambers of piglet intestinal sections fed diets varying in B-glucan content. Indicated as mean mannitol flux (nm/cm/hr) with error bars representing SEM. Fluxes that do not share a common letter are significantly different ($p<0.05$).

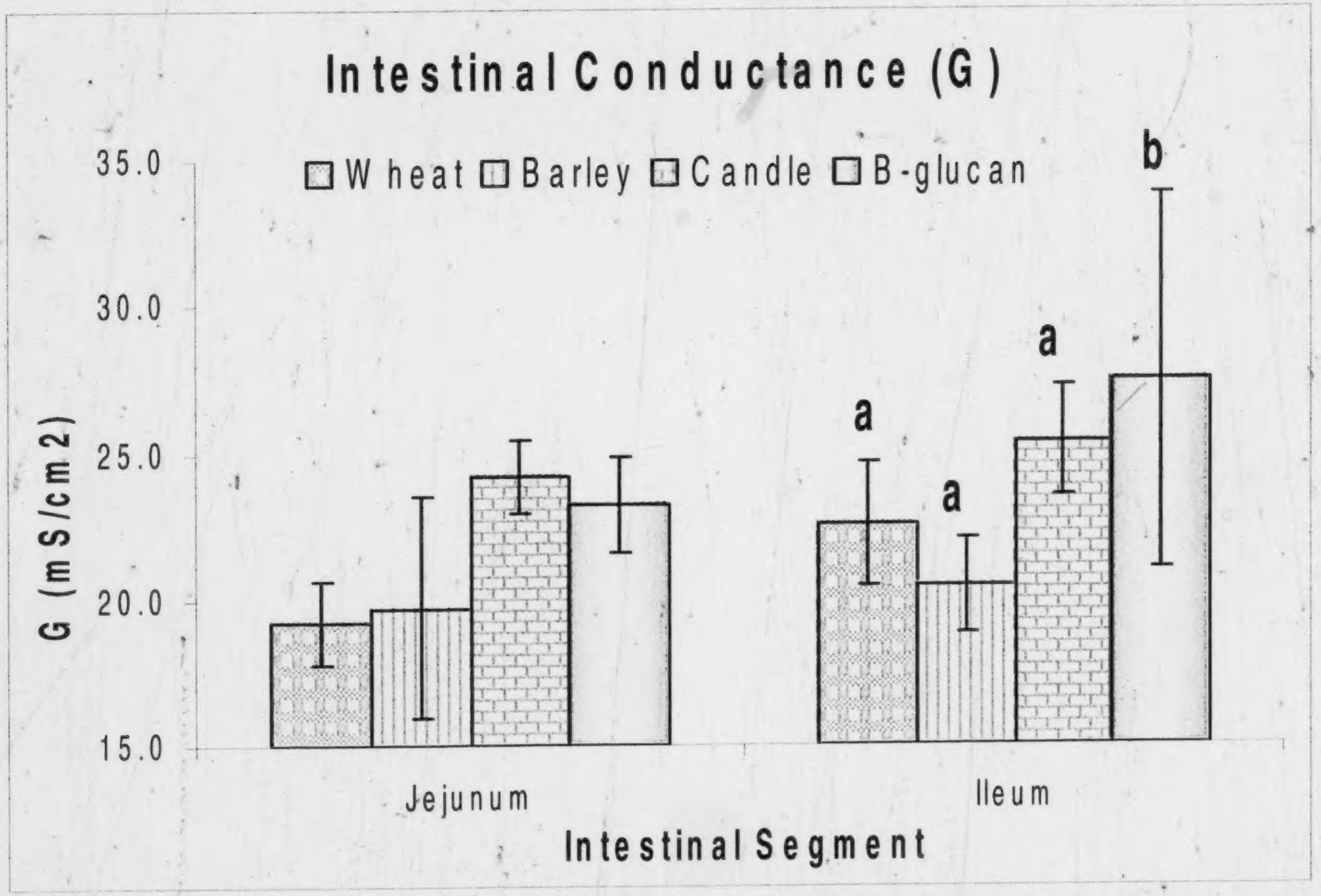


Figure 7. Intestinal G measured in Ussing Chambers of piglet intestinal sections fed diets varying in B-glucan content. Indicated as mean G (mS/cm^2) with error bars representing SEM. Mean G that do not share a common letter are significantly different ($p<0.05$).

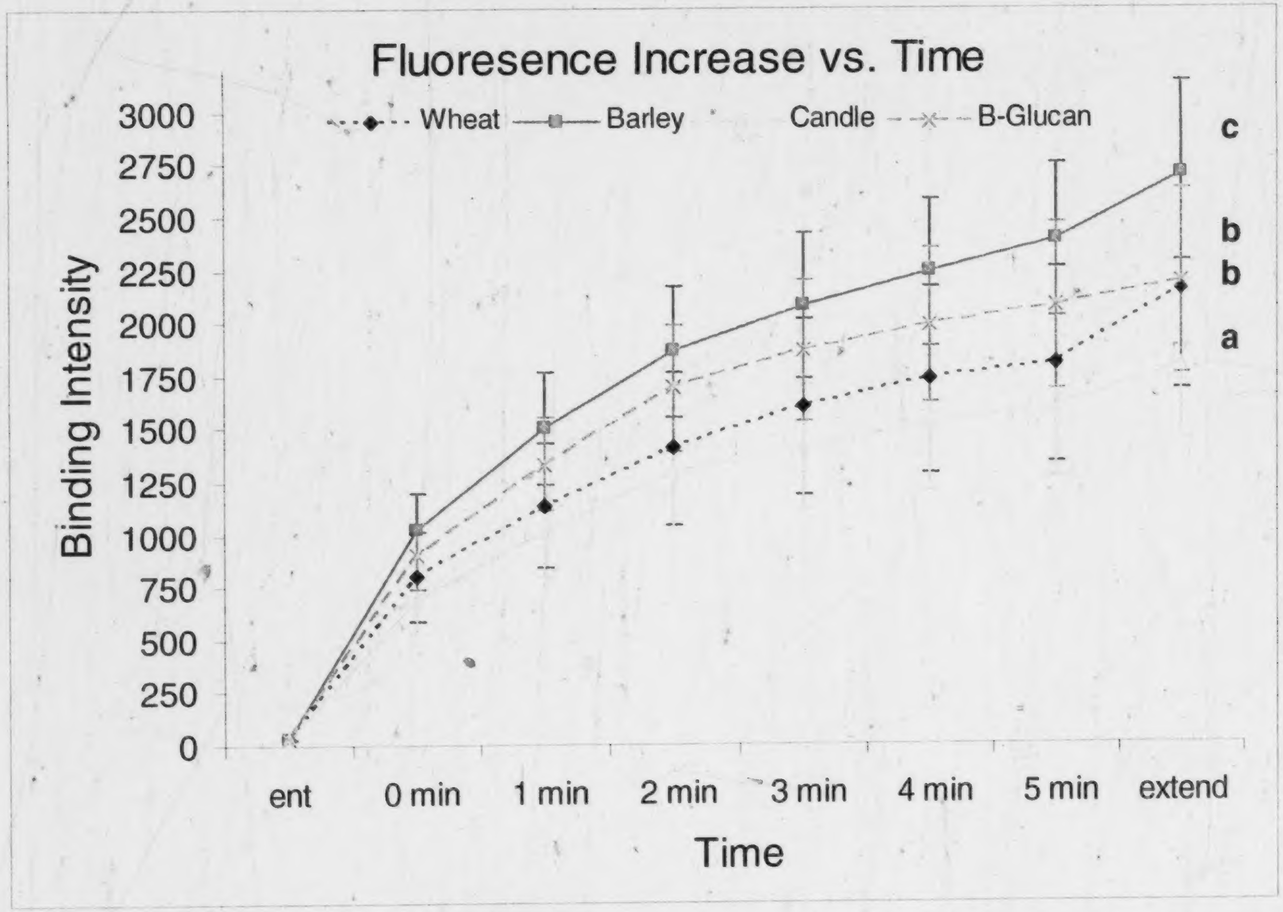


Figure 8. Binding Intensity comparison of ETEC Adhesion assay with fresh enterocytes from piglets fed varying levels of B-glc. Adhesion assay data was analyzed for significance across the binding intensity line with a repeated measures Anova design. Mean values not sharing the same letter are significantly different ($p<0.05$).

6.5 REFERENCES

Abel,G. and Czop,J.K. 1992. Stimulation of human monocyte beta-glucan receptors by glucan particles induces production of TNF-alpha and IL-1 beta. Int. J. Immunopharmacol. 14: 1363-1373.

Anderson,J.W., Story,L., Sieling,B., Chen,W.J., Petro,M.S., and Story,J. 1984. Hypocholesterolemic effects of oat-bran or bean intake for hypercholesterolemic men. Am. J. Clin. Nutr. 40: 1146-1155.

Au,B.T., Williams,T.J., and Collins,P.D. 1994. Zymosan-induced IL-8 release from human neutrophils involves activation via the CD11b/CD18 receptor and endogenous platelet-activating factor as an autocrine modulator. J. Immunol. 152: 5411-5419.

Blood,D.C. and Rodostits,O.M. 1989. Veterinary Medicine: A textbook of the diseases of cattle, sheep, pigs, goats and horses. 7th Ed. Baillière Tindall, Toronto. pp 1502.

Braakman,E., Sturm,E., Vijverberg,K., van Krimpen,B.A., Gratama,J.W., and Bolhuis,R.L. 1991. Expression of CD45 isoforms by fresh and activated human gamma delta T lymphocytes and natural killer cells. Int. Immunol. 3: 691-697.

Braaten,J.T., Wood,P.J., Scott,F.W., Riedel,K.D., Poste,L.M., and Collins,M.W. 1991. Oat gum lowers glucose and insulin after an oral glucose load. Am. J. Clin. Nutr. 53: 1425-1430.

Braaten,J.T., Wood,P.J., Scott,F.W., Wolynetz,M.S., Lowe,M.K., Bradley-White,P., and Collins,M.W. 1994. Oat beta-glucan reduces blood cholesterol concentration in hypercholesterolemic subjects. Eur. J. Clin. Nutr. 48: 465-474.

Breves,G., Szentkuti,L., and Schroder,B. 2001. Effects of oligosaccharides on functional parameters of the intestinal tract of growing pigs. Dtsch. Tierarztl. Wochenschr. 108: 246-248.

Brown,G.D. and Gordon,S. 2001. Immune recognition. A new receptor for beta-glucans. Nature 413: 36-37.

Brown,G.D. and Gordon,S. 2003. Fungal beta-glucans and mammalian immunity. Immunity. 19: 311-315.

Brown,G.D., Herre,J., Williams,D.L., Willment,J.A., Marshall,A.S., and Gordon,S. 2003. Dectin-1 mediates the biological effects of beta-glucans. J. Exp. Med. 197: 1119-1124.

Brown,K.H., Perez,F., Peerson,J.M., Fadel,J., Brunsgaard,G., Ostrom,K.M., and MacLean,W.C., Jr. 1993. Effect of dietary fiber (soy polysaccharide) on the severity, duration, and nutritional outcome of acute, watery diarrhea in children. Pediatrics 92: 241-247.

Buddington,R.K. 1998. The influences of dietary inputs on the neonatal gastrointestinal tract: Managing the development of a complex ecosystem. J.Anim.Feed.Sci. 7 (suppl. 1): 155-165.

Chen,J.T. and Hasumi,K. 1993. Activation of peritoneal macrophages in patients with gynecological malignancies by sizofiran and recombinant interferon-gamma. Biotherapy 6: 189-194.

Chihara,G. 1992. Recent progress in immunopharmacology and therapeutic effects of polysaccharides. Dev. Biol. Stand. 77: 191-197.

Clarke,J.M. and Morton,R.J. 2000. Development of an in vitro fluorometric assay to study adherence of Pasteurella haemolytica to bovine cells. Am. J. Vet. Res. 61: 129-132.

Correa-Matos,N.J., Donovan,S.M., Isaacson,R.E., Gaskins,H.R., White,B.A., and Tappenden,K.A. 2003. Fermentable fiber reduces recovery time and improves intestinal function in piglets following Salmonella typhimurium infection. J. Nutr. 133: 1845-1852.

Decuypere,N., Dierick,N., and Boddez,S. 1998. The potentials of immunostimulatory substances (á-1,3/1,6 glucans) in pig nutrition. J.Anim.Feed.Sci. 7, 259.

Di Luzio,N.R., McNamee,R.B., Williams,D.L., Gilbert,K.M., and Spanjers,M.A. 1979a. Glucan induced inhibition of tumor growth and enhancement of survival in a variety of transplantable and spontaneous murine tumor models. Adv. Exp. Med. Biol. 121: 269-290.

Di Luzio,N.R., Williams,D.L., McNamee,R.B., Edwards,B.F., and Kitahama,A. 1979b. Comparative tumor-inhibitory and anti-bacterial activity of soluble and particulate glucan. Int. J. Cancer 24: 773-779.

Doita,M., Rasmussen,L.T., Seljelid,R., and Lipsky,P.E. 1991. Effect of soluble aminated beta-1,3-D-polyglucose on human monocytes: stimulation of cytokine and prostaglandin E2 production but not antigen-presenting function. J. Leukoc. Biol. 49: 342-351.

Dongowski,G., Huth,M., Gebhardt,E., and Flamme,W. 2002. Dietary fiber-rich barley products beneficially affect the intestinal tract of rats. J. Nutr. 132: 3704-3714.

Dritz,S.S., Shi,J., Kielian,T.L., Goodband,R.D., Nelssen,J.L., Tokach,M.D., Chengappa,M.M., Smith,J.E., and Blecha,F. 1995. Influence of dietary beta-glucan on growth performance, nonspecific immunity, and resistance to Streptococcus suis infection in weanling pigs. J. Anim Sci. 73: 3341-3350.

Egeli,A.K. and Framstad,T. 1999. An evaluation of iron-dextran supplementation of piglets administered by injection on the first, third or fourth day after birth. Res. Vet. Sci. 66: 179-184.

Estrada,A., Yun,C.H., Van Kessel,A., Li,B., Hauta,S., and Laarveld,B. 1997. Immunomodulatory activities of oat beta-glucan in vitro and in vivo. Microbiol Immunol 41: 991-998.

Fan,M.Z., Stoll,B., Jiang,R., and Burrin,D.G. 2001. Enterocyte digestive enzyme activity along the crypt-villus and longitudinal axes in the neonatal pig small intestine. J. Anim Sci. 79: 371-381.

Field,C.J., McBurney,M.I., Massimino,S., Hayek,M.G., and Sunvold,G.D. 1999. The fermentable fiber content of the diet alters the function and composition of canine gut associated lymphoid tissue. Vet. Immunol. Immunopathol. 72: 325-341.

Fortin,A., Robertson,W.M., Kibite,S., and Landry,S.J. 2003. Growth performance, carcass and pork quality of finisher pigs fed oat-based diets containing different levels of beta-glucans. J. Anim Sci. 81: 449-456.

Grange,P.A. and Mouricout,M.A. 1996. Transferrin associated with the porcine intestinal mucosa is a receptor specific for K88ab fimbriae of Escherichia coli. Infect. Immun. 64: 606-610.

Hashimoto,T., Ohno,N., Adachi,Y., and Yadomae,T. 1997. Nitric oxide synthesis in murine peritoneal macrophages by fungal beta-glucans. Biol Pharm Bull 20: 1006-1009.

Hiss,S. and Sauerwein,H. 2003. Influence of dietary ss-glucan on growth performance, lymphocyte proliferation, specific immune response and haptoglobin plasma concentrations in pigs. J. Anim Physiol Anim Nutr. (Berl) 87: 2-11.

Hoffman,O.A., Olson,E.J., and Limper,A.H. 1993. Fungal beta-glucans modulate macrophage release of tumor necrosis factor-alpha in response to bacterial lipopolysaccharide. Immunol. Lett. 37: 19-25.

Izydorczyk,M.S., Storsley,J., Labossiere,D., MacGregor,A.W., and Rossnagel,B.G. 2000. Variation in total and soluble beta-glucan content in hulless barley: effects of thermal, physical, and enzymic treatments. J. Agric. Food Chem. 48: 982-989.

Jenkins,D.J., Wolever,T.M., Rao,A.V., Hegele,R.A., Mitchell,S.J., Ransom,T.P., Boctor,D.L., Spadafora,P.J., Jenkins,A.L., Mehling,C., and . 1993. Effect on blood lipids of very high intakes of fiber in diets low in saturated fat and cholesterol. N. Engl. J. Med. 329: 21-26.

Jiang,G. and Vasanthan,T. 2000. MALDI-MS and HPLC quantification of oligosaccharides of lichenase-hydrolyzed water-soluble beta-glucan from ten barley varieties. J. Agric. Food Chem. 48: 3305-3310.

Jin,L.Z. and Zhao,X. 2000. Intestinal receptors for adhesive fimbriae of enterotoxigenic Escherichia coli (ETEC) K88 in swine--a review. Appl. Microbiol. Biotechnol. 54: 311-318.

Judd,P.A. and Truswell,A.S. 1981. The effect of rolled oats on blood lipids and fecal steroid excretion in man. Am. J. Clin. Nutr. 34: 2061-2067.

Kaufhold,J. Hammon,H.M., and Blum,J.W. 2000. Fructo-oligosaccharide supplementation: effects on metabolic, endocrine and hematological traits in veal calves. J. Vet. Med. - Series A. 47: 17-29.

Kim,H.M., Han,S.B., Oh,G.T., Kim,Y.H., Hong,D.H., Hong,N.D., and Yoo,I.D. 1996. Stimulation of humoral and cell mediated immunity by polysaccharide from mushroom Phellinus linteus. International Journal of Immunopharmacology 5: 303-?

Kirby,R.W., Anderson,J.W., Sieling,B., Rees,E.D., Chen,W.J., Miller,R.E., and Kay,R.M. 1981. Oat-bran intake selectively lowers serum low-density lipoprotein cholesterol concentrations of hypercholesterolemic men. Am. J. Clin. Nutr. 34: 824-829.

Komatsu,N., Okubo,S., Kikumoto,S., Kimura,K., and Saito,G. 1969. Host-mediated antitumor action of schizophyllan, a glucan produced by Schizophyllum commune. Gann 60: 137-144.

Laux,D.C., McSweegan,E.F., Williams,T.J., Wadolkowski,E.A., and Cohen,P.S. 1986. Identification and characterization of mouse small intestine mucosal receptors for Escherichia coli K-12(K88ab). Infect. Immun. 52: 18-25.

Lia,A., Hallmans,G., Sandberg,A.S., Sundberg,B., Aman,P., and Andersson,H. 1995. Oat beta-glucan increases bile acid excretion and a fiber-rich barley fraction increases cholesterol excretion in ileostomy subjects. Am. J. Clin. Nutr. 62: 1245-1251.

Lim,B.O., Yamada,K., Nonaka,M., Kuramoto,Y., Hung,P., and Sugano,M. 1997. Dietary fibers modulate indices of intestinal immune function in rats. J. Nutr. 127: 663-667.

McIntosh,G.H., Whyte,J., McArthur,R., and Nestel,P.J. 1991. Barley and wheat foods: influence on plasma cholesterol concentrations in hypercholesterolemic men. Am. J. Clin. Nutr. 53: 1205-1209.

Mineo,H., Amano,M., Chiji,H., Shigematsu,N., Tomita,F., and Hara,H. 2004. Indigestible disaccharides open tight junctions and enhance net calcium, magnesium, and zinc absorption in isolated rat small and large intestinal epithelium. Dig. Dis. Sci. 49: 122-132.

Mineo,H., Hara,H., Shigematsu,N., Okuhara,Y., and Tomita,F. 2002. Melibiose, difructose anhydride III and difructose anhydride IV enhance net calcium absorption in rat small and large intestinal epithelium by increasing the passage of tight junctions in vitro. J. Nutr. 132: 3394-3399.

NRC 1998. Nutrient Requirements of swine (10th Ed.). Subcom. Swine Nutr., Comm Anim. Nutr., Board Agric., NRC. Nat. Acad. Press, Wash. pp 192.

Ohno,N., Hashimoto,T., Adachi,Y., and Yadomae,T. 1996. Conformation dependency of nitric oxide synthesis of murine peritoneal macrophages by beta-glucans in vitro. Immunol. Lett. 53: 157-163.

Olson,E.J., Standing,J.E., Griego-Harper,N., Hoffman,O.A., and Limper,A.H. 1996. Fungal beta-glucan interacts with vitronectin and stimulates tumor necrosis factor alpha release from macrophages. Infection & Immunity 64: 3548-3554.

Rankin,J.A., Sylvester,I., Smith,S., Yoshimura,T., and Leonard,E.J. 1990. Macrophages cultured in vitro release leukotriene B4 and neutrophil attractant/activation protein (interleukin 8) sequentially in response to stimulation with lipopolysaccharide and zymosan. J. Clin. Invest 86: 1556-1564.

Rasmussen,L.T., Lipsky,P.E., and Seljelid,R. 1987. Production of prostaglandin E2 and interleukin 1 by mouse peritoneal macrophages stimulated with beta-1,3-D-glucan derivatized plastic beads. Scand. J. Immunol. 26: 731-736.

Riggi,S.J. and Di Luzio,N.R. 1961. Identification of a reticuloendothelial stimulating agent in zymosan. Am. J. Physiol 200: 297-300.

Ross,G.D., Cain,J.A., Myones,B.L., Newman,S.L., and Lachmann,P.J. 1987. Specificity of membrane complement receptor type three (CR3) for beta-glucans. Complement 4: 61-74.

Sakurai,T., Ohno,N., and Yadomae,T. 1994. Changes in immune mediators in mouse lung produced by administration of soluble (1-->3)-beta-D-glucan. Biol. Pharm. Bull. 17: 617-622.

Sarmiento,J.I., Casey,T.A., and Moon,H.W. 1988. Postweaning diarrhea in swine: experimental model of enterotoxigenic Escherichia coli infection. Am. J. Vet. Res. 49: 1154-1159.

Schley,P.D. and Field,C.J. 2002. The immune-enhancing effects of dietary fibres and prebiotics. Br. J. Nutr. 87 Suppl 2: S221-S230.

Schoenherr,W.D., Pollmann,D.S., and Coalson,J.A. 1994. Titration of MacroGard-S on growth performance of nursery pigs. J.Anim Sci. 72 (suppl. 2), 57.

Sherwood,E.R., Varma,T.K., Fram,R.Y., Lin,C.Y., Koutrouvelis,A.P., and Toliver-Kinsky,T.E. 2001. Glucan phosphate potentiates endotoxin-indued interferon-γ expression in immunocompetent mice, but attenuates induction of endotoxin tolerance. Clin. Sci. 101: 541-550.

Sherwood,E.R., Williams,D.L., McNamee,R.B., Jones,E.L., Browder,I.W., and Di Luzio,N.R. 1987. Enhancement of interleukin-1 and interleukin-2 production by soluble glucan. Int. J. Immunopharmacol. 9: 261-267.

Sherwood,E.R., Williams,D.L., McNamee,R.B., Jones,E.L., Browder,I.W., and DI LUZIO,N.R. 1988. Soluble glucan and lymphokine-activated killer (LAK) cells in the therapy of experimental hepatic metastases. J. Biol. Response Mod. 7: 185-198.

Slavin,J.L., Nelson,N.L., McNamara,E.A., and Cashmere,K. 1985. Bowel function of healthy men consuming liquid diets with and without dietary fiber. JPEN J. Parenter. Enteral Nutr. 9: 317-321.

Thorns,C.J., Sojka,M.G., and Roeder,P.L. 1989. Detection of fimbrial adhesins of ETEC using monoclonal antibody-based latex reagents. Vet. Rec. 125: 91-92.

Tungland,B.C. and Meyer,D. 2002. Nondigestible Oligo- and Polysaccharides (Dietary Fiber): their physiology and role in human health and food. Comp. Rev. Food Science & Food Safety 3: 73-92.

Vanderhoof,J.A., Murray,N.D., Paule,C.L., and Ostrom,K.M. 1997. Use of soy fiber in acute diarrhea in infants and toddlers. Clin. Pediatr. (Phila) 36: 135-139.

Velazquez,O.C., Lederer,H.M., and Rombeau,J.L. 1997. Butyrate and the colonocyte: production, absorption, and treatment of hyperlipidaemias. In: Dietary Fibre - A Component of Food. Schweizer,T.F. and Edwards,C.A. eds. Springer-Verlag, London, UK. pp 295-332.

Williams,D.L. and Di Luzio,N.R. 1979. Glucan induced modification of experimental Staphylococcus aureus infection in normal, leukemic and immunosuppressed mice. Adv. Exp. Med. Biol. 121: 291-306.

Willment,J.A., Gordon,S., and Brown,G.D. 2001. Characterization of the human β-blucan receptor and its alternatively spliced isoforms. J Biol Chem 276: 43818-43823.

Zhang,J.X., Hallmans,G., Andersson,H., Bosaeus,I., Aman,P., Tidehag,P., Stenling,R., Lundin,E., and Dahlgren,S. 1992. Effect of oat bran on plasma cholesterol and bile acid excretion in nine subjects with ileostomies. Am. J. Clin. Nutr. 56: 99-105.

Zhang,K. and Petty,H.R. 1994. Influence of polysaccharides on neutrophil function: specific antagonists suggest a model for cooperative saccharide-associated inhibition of immune complex-triggered superoxide production. J. Cell Biochem. 56: 225-235.

Chapter 7 – SUMMARY AND FUTURE CONSIDERATIONS

7.1 SUMMARY

To summarize the major hypotheses and findings from this thesis:

1. It was hypothesized that oral inoculation with live Enterotoxigenic *Escherichia coli* (ETEC) to weaned piglets will result in reproducible clinical symptoms of mild infection.

In Chapter 3, infection trials resulted in a great deal of variability in the severity of illness and it was not possible to develop a reproducible model of ETEC infection in newly weaned piglets.

2. It was hypothesized that an *in vivo* loop model would demonstrate the early signs of ETEC infection.

The increased mannitol permeability (ManP) and conductance (G) that was found in tissues that were exposed to ETEC suggested that early signs of infection occurred and that there was an influence on tight junction integrity. Therefore, the early stages of ETEC infection were detected in this model. Immune results obtained in Chapter 3 demonstrated that the surgical procedure did not influence peripheral blood phenotype or the ability of these cells to incorporate ^{3}H-thymidine (*ex vivo* and following mitogen stimulation). Mesenteric Lymph Nodes (MLN) were also isolated post-surgery and were shown to incorporate ^{3}H-thymidine at an appreciable rate. Results presented in this chapter support the use of the *in situ* surgical model for determining the influence of dietary nutrients on the early stages of ETEC infection.

4. It was hypothesized that piglet innate immune defense at 35 days is more developed than at 21 days.

In Chapter 4, the ability of neutrophils to respond faster or at greater magnitude to a challenge in vitro by increasing their oxidative burst did not differ between 21 and 35 days of age. It can be concluded that this immune function appears to be well developed at weaning.

5. It was also hypothesized that piglet adaptive immune defense at 35 days is more developed than at 21 days.

Several immune parameters were used in Chapter 4 to measure adaptive immune function in young piglets. This study contributes to the literature by characterizing the changes that occur in this tissue with age, specifically decreases in antigen naïve cells (Cluster of Differentiation (CD) 45RA+) and the increase in the immature population of PPBcell+ cells. The observed increases in lymphocyte incorporation of ^{3}H-thymidine in peripheral blood lymphocytes (PBL; to phytohemagglutinin (PHA) and pokeweed mitogen (PWM) and MLN (to PHA, PWM and lipopolysaccharide (LPS)) suggests that adaptive immune function increases with age in these animals in both blood and a tissue in close proximity to the intestine. This was further supported by an increased rate of interleukin (IL)-2 production in stimulated MLN. Phenotype data also supports the hypothesis of an increase in adaptive immune function with age, as MLN had an increased proportion of total B cells and Immunoglobulin (Ig) A+ cells, whereas PBL had an increased proportion of T and B cell expression of an adhesion molecule (CD44). Taken together, these results suggest an improvement in adaptive immune function with age.

6. It was hypothesized that supplementation of dietary glutamine (gln) to newly weaned piglets would improve innate immune defense.

The increases in neutrophil granularity of gln-fed piglets in Chapter 5 suggest an improvement in innate immune defense. However, since no influence on oxidative burst was found in these animals, the physiological relevance with respect to infection defense is not clear.

7. It was also hypothesized that supplementation of dietary gln to newly weaned piglets will improve adaptive immune defense.

In Chapter 5, it was demonstrated that feeding gln influences several components of the adaptive immune system. In MLN, there was a significantly higher response (stimulation index) to PWM and LPS in gln-fed piglets, while PP lymphocytes of gln-fed piglets had a significantly higher response to PHA (^{3}H-thymidine) and to LPS (stimulation index). Feeding gln resulted in a lower production of Th1-type cytokines following stimulation with PHA, in PBL, and a lower production of both Th1 (IL-2) and Th2 (IL-4) cytokines in MLN compared to cells from ctl-fed animals. Although feeding gln to piglets resulted in only minor changes on the proportion of the major immune cell populations in these tissues, there were significantly lower proportions of antigen naïve cells in PBL and MLN of gln-fed piglets. Although there is considerable literature which has demonstrated the ability of oral gln to influence immune function in immunosuppressed conditions, relatively few were seen in the current study. This may have been influenced by species differences in the current study. In addition, all piglets were healthy and consuming diets that contained an adequate amount of gln, which may have blunted the effects of gln on immune function.

8. It was hypothesized that isolated intestinal loops challenged with ETEC from gln-supplemented piglets will maintain intestinal barrier function and functional integrity.

Measurement of luminal to serosal mannitol flux demonstrated that feeding gln to newly weaned piglets did not influence intestinal barrier function in ETEC inoculated loops. However, gln did influence intestinal ion movement in ETEC-inoculated loops. Results in Chapter 5 demonstrated that provision of dietary gln resulted in a significantly lower potential difference (PD) and short-circuit current (Isc) in ETEC-inoculated loops. While gln has been shown to stimulate ion absorption previously (Rhoads 1991), the results of the present study demonstrated that feeding gln could influence these *in vitro* measurements. Future studies are warranted to determine the mechanisms by which gln influences ion movement, whether by increasing absorption, preventing secretion, or influencing expression of proteins in mucosal cells.

9. Supplementation of dietary barley-derived beta-glucan (B-glc) will improve innate immune defense.

In Chapter 6, there was no effect of feeding different levels of B-glc on the ability of peripheral blood neutrophils to produce free radicals when stimulated *in vitro*. This finding in an animal feeding model is inconsistent with studies in the literature, where B-glc benefited innate immune function when neutrophils and macrophages were incubated with B-glc *in vitro* or provided intravenously.

10. Supplementation of barley-derived B-glc can improve adaptive immune defense.

In PBL, the percentage of CD8+ cells expressing CD45RA was significantly higher in wheat and Candle-fed piglets. Piglets consuming the high B-glc diet had a higher concentration of white blood cells and lymphocytes. The physiological effect of a higher leukocyte concentration (within the normal range) requires further study. The results of Chapter 6 did not support an effect of feeding B-glc on immune function.

11. Supplementation of barley-derived B-glc can benefit gut development in newly weaned piglets

In Chapter 6, tissues of piglets fed the diet high in B-glc demonstrated a higher ileal mannitol flux and conductance in the B-glc group. Although this might be considered a negative effect with respect to bacterial defense, there were no changes in the proportion of antigen mature cells in PP or MLN, which would be expected if there was a change in gut barrier function. Additionally, no differences were found in the ability of immune cells from B-glc fed piglets to respond to mitogens. This suggests that these changes in permeability did not result in significant antigen exposure to GALT.

12. Supplementation of diets varying in fiber and B-glc composition influences the ability of isolated enterocytes to bind fluorescein isothiocyanate- (FITC) labeled *Escherichia coli.*

The ETEC adhesion assay described in Chapter 6 demonstrated that there was not an inverse relationship between the b-glc content of the diet and the rate of *E. coli* binding to isolated enterocytes. The higher rate of binding to labeled *E.coli* by epithelial cells from piglets fed the barley diet may have resulted from the presence of a dietary nutrient other than B-glc. Also of interest was the higher rate of binding when B-glc was added to the Candle diet. This requires future study in an *in vivo* model. However, it must be stressed that this in vitro assay eliminates the contribution of luminal factors such as gut microorganisms, immune cells and the protective mucus layer. Therefore, these findings need to be followed up in a more physiological in situ model, such as the one described in Chapter 3.

7.2 FUTURE CONSIDERATIONS

Piglet Development

Early weaning of piglets is not only strenuous to the piglets' immune system, but the resulting growth lag and occasional illness is also problematic to the industry. Therefore, a great deal of effort has been used to specially design weaning diets to maximize growth. More recently, piglet diets are being designed to improve immune function as well. Unfortunately, there have been few experimental studies of immune function in swine, possibly due to the cost and the limited number of swine-specific immune markers that are commercially available. Chapter 4 contributes to the literature by providing a link between the changes to immune cell subpopulations and function that occur during the early weaning period. Of interest to comparative immunology are the reasons why piglets have evolved differently from other species in function and immune cell populations, specifically the double positive CD4CD8 T cells. This ability of this unique population to deliver both helper and cytotoxic/suppressor signals is a novel concept in immunology and warrants further investigation. There are also differences in the lymphocyte migration patterns of piglets compared to other species. The reasons for these differences in morphology are not known, but activated lymphocytes entering the circulatory system more quickly might enable piglets to respond more quickly when antigens are encountered. Swine producers define an optimal time of weaning in order to minimize losses and optimize growth. Perhaps a novel concept would be to consider an optimal time of weaning, based on immune development/maturation. Results presented in this thesis show an improvement in adaptive immune function between 21 and 35 days of age. This suggests that early weaning places a piglet at an additional risk and provides support for delaying the time of weaning in piglets.

Immunonutrients

The ability of naturally occurring nutrients, the immunonutrients, to influence immune function has received a great deal of attention in recent years. However, this should come as no surprise as humans have been benefiting from these abilities for centuries by consuming these nutrients in the foods they eat. This was long before the advent of pharmaceuticals and immunomodulators designed to improve immune function.

Gln is an amino acid of many names, having been referred to as conditionally essential (Lacey & Wilmore 1990), an immunonutrient (Field et al. 2000), a life-saving nutrient (Preiser & Wernerman 2003), a magic bullet (Duggan 1998), and the gas pedal (but not the Ferrari: Rhoads 2004). Based on the abundance of experimental studies, it is generally accepted that the amino acid gln has an influence on immune function. The gut is a major gln consumer, utilizing almost all that is absorbed from the lumen (Wu 1998) while extracting circulating gln from the bloodstream (Souba 1993). Thus, the extent to which dietary gln can influence peripheral immune sites requires more attention. This is especially important in light of the findings from Chapter 5 in which feeding gln was shown to influence peripheral immune cell function and maturation (neutrophil granularity, cytokine production, CD45 expression). Unfortunately, it was not possible to determine the mechanisms for these peripheral effects in the present study. The ability of gln to influence ion transport in ETEC-inoculated intestinal loops was also demonstrated in Chapter 5. Gln has been shown to stimulate ion transport in infected intestinal tissues of piglets *in vitro* (Rhoads et al. 1991). The current study supports this finding in a more physiological model. Based on the results presented in this thesis, provision of gln in the diet of healthy piglets would enable the immune systems of these animals to respond more effectively to pathogenic challenge and may maintain absorption during enteric infections.

The history associated with B-glc is equally interesting, as these have come full circle from being considered as a major anti-nutritional factor, to being proposed as an immunonutrient (based on *ex vivo* supplementation). Despite the abundant evidence from *in vitro* studies, it appears that increasing the B-glc content of the weaning piglet diet has limited effects on the immune system. However, the majority of these *in vitro* immune studies assume that this compound will reach the immune cells, and there is no evidence to suggest that b-glc enter the body intact. Future studies are warranted to examine the extent to which B-glc can contact the immune tissues and cells of GALT. If specific reagents are not available for identifying B-glc, designing a method that would enable quantification of B-glc or their metabolites in immune tissues might answer these questions. It would also be interesting to determine whether the structure of the B-glc themselves (i.e. 1,3/1,4 vs 1,3/1,6) are responsible for some of the observed effects. From the results shown in Chapter 6, the concentration of B-glc in the diet influences the concentration of peripheral immune cells in a direct manner by a yet to be determined mechanism. Determining the B-glc metabolites which cross the gut barrier and influence peripheral immune function may reveal some interesting findings. Results from Chapter 6 also demonstrated that the distal ileum is a site of major importance in the mode of action of B-glc. The immune cell populations in the adjacent PP were also characterized at this site, as was their ability to incorporate ^{3}H-thymidine *ex vivo* and when stimulated with several mitogens. It also seems hardly coincidental that this is also the site colonized by microorganisms, actively producing short-chain fatty acids (SCFA) by fermentation. A new focus for B-glc studies would be to further characterize the interactions between the luminal microflora, the mucosa, and the underlying GALT tissues in this area of the gut.

ETEC and Gut Development

Antibiotics are efficient in reducing mortality from clinical disease and most starter diets formulated for swine contain antibiotics (Patience & Thacker 1989). Major efforts to overcome infectious diseases have focused on the development of stronger antibiotics to defeat resistant pathogenic microorganisms (Kawakami 2003). However, the agriculture industry is facing increased pressure to cut back on antibiotic use. One of the potential applications of the findings of this thesis would be to suggest nutrients that might benefit the developing immune system so as to reduce the need for antibiotics. In Chapter 6, mucosal cells isolated from barley-fed piglets were shown to bind ETEC with a greater intensity than all of the other groups. Also, mucosal cells from B-glc-fed piglets had a higher binding intensity than Candle group and these diets differed only in the added B-glc supplement. However, what could not be determined were the dietary components responsible for these influences. The diet-induced changes in ETEC binding do suggest that it might be possible to formulate diets that would minimize the ability of ETEC to bind to mucosal cells *in vivo*. While addition of B-glc to the diet may not have prevented binding, defining whether dietary fiber or potentially other nutrients influences ETEC binding requires further study. There were also encouraging results in this thesis that dietary gln influences ion movement in ETEC-exposed mucosal tissues. This suggests that it may be possible to include gln in the diet of young piglets, not only for prevention of ETEC infections, but as a potential therapy if they do become ill However, a more detailed analysis of expression of intracellular mediators and tight junction proteins following ETEC exposure is warranted in future studies.

Neonatal Models

In considering the results and ideas generated in this thesis, it must be stressed that the findings may be considered for species other than swine. Many of these studies were conducted not only to advance the knowledge of swine immune function, but also with the concept of using pigs as a model for human infants. There are many similarities between these two organisms and pigs possess several advantages to mice, which are the most frequently used experimental model. Although utilization of gln in infant formulas has not received universal acceptance, it has been suggested that some of the results of recent pediatric clinical gln trials may change this (Rhoads 2004). There already exists physiologic rationale to support the addition of dietary fiber to infant formulas (Correa-Matos et al. 2003) and this research could be extended to include B-glc as a potential source of fiber.

Practical Considerations

The complex field of swine nutrition has evolved from the more conventional "feed and weigh" studies to a more detailed analysis of the metabolic and cellular consequences of diet and specific nutrients. Reduced costs of synthetic amino acids means that more options will be available for balancing diets, and the dependency on conventional protein sources such as soybean meal and canola meal will diminish, as will the costly waste of excess nutrients (Patience & Thacker 1989). One of the goals of this thesis was to design evidence-based recommendations for inclusions of specific levels of immunonutrients into conventional diets of weanling piglets. Now that scientific evidence is available for the use of these nutrients, the challenge is attempting to incorporate this knowledge into common practice. A potential obstacle is that producers must consider cost benefits in making their decisions. However, recent events in agriculture have resulted in an increased consumer awareness of the importance of well-defined dietary ingredients. Therefore, the natural properties of immunonutrients already provide an appealing option to producers. The challenge will be to provide evidence that the cost investment with such nutrients results in added gains (i.e. increase in growth, reduced mortality, reduced morbidity). Evidence has been presented in this thesis that inclusion of gln in piglet diets can benefit immune function and potentially protect against infections. However, questions remain about the optimal level of inclusion of gln, which was selected at 4.4% w/w based on previous studies, mostly conducted in stressed animals. With no previous frame of reference for inclusion of Barley-derived 1,3/1,4 B-glc, several increasing increments were formulated to determine an effect. Although there were limited findings with these nutrients, additional studies are necessary before ruling out the benefits for inclusion of B-glc in swine diets. In addition, the number of animals used in these trials was relatively small from a production perspective and the feeding period was relatively short. Therefore, additional trials, with different inclusion rates and/or combinations of these nutrients and conducted for longer periods of time would be necessary to convince producers to use immunonutrients such as gln and B-glc in swine diets.

7.3 REFERENCES

Correa-Matos,N.J., Donovan,S.M., Isaacson,R.E., Gaskins,H.R., White,B.A., and Tappenden,K.A. 2003. Fermentable fiber reduces recovery time and improves intestinal function in piglets following Salmonella typhimurium infection. J. Nutr. 133: 1845-1852.

Duggan,C. 1998. Glutamine-based oral rehydration solutions.: the magic bullet revisited? J. Ped. Gastr. Nutr. 26: 533-535.

Field,C.J., Johnson,I., and Pratt,V.C. 2000a. Glutamine and arginine: immunonutrients for improved health. Med. Sci. Sports Exerc. 32: S377-S388.

Kawakami,K. 2003. Promising immunotherapies with Th1-related cytokines against infectious diseases. J. Infect. Chemother. 9: 201-209.

Lacey,J.M. and Wilmore,D.W. 1990. Is glutamine a conditionally essential amino acid? Nutr. Rev. 48: 297-309.

Patience,J.F. and Thacker,P.A. 1989. Swine Nutrition Guide. Univ. Saskat. and Prairie Swine Ctr, Saskatoon, Sask. pp 260.

Preiser,J. and Wernerman,J. 2003. Glutamine, a life-saving nutrient, but why? Crit. Care Med. 31: 2555-.

Rhoads,J.M., Keku,E.O., Quinn,J., Woosely,J., and Lecce,J.G. 1991. L-glutamine stimulates jejunal sodium and chloride absorption in pig rotavirus enteritis. Gastroenterology 100: 683-691.

Rhoads,M. 2004. Glutamine is the gas pedal but not the Ferrari. J. Ped. Gastr. Nutr. 38: 474-476.

Souba,W.W. 1993. Intestinal glutamine metabolism and nutrition. Journal of Nutritional Biochemistry 4: 2-9.

Wu,G. 1998. Intestinal mucosal amino acid catabolism. J Nutr 128: 1249-1252.

APPENDIX

TABLE 1. Proximate analysis of major feed ingredients for dietary B-glucan supplementation trials

Sample	% Moisture	% B-Glucan[1]	% Starch	% Protein	% Lipids	% Ash	% soluble dietary fiber	% insoluble dietary fiber	% total dietary fiber
Candle Barley[2]	3.14	7.25	71.90	9.4	0.83	0.57			
Barley	8.75	3.30	48.98	13.59	2.18	3.74			
Soybean	7.86	0.23	5.59	46.89	2.73	7.12			
Wheat	9.72	1.13	53.61	17.01	2.45	2.61			
Candle Fiber Concentrate		54.52	12.77	6.46	0	0.73	55.07	17.32	72.39
Candle Crude Starch	n.a.	n.a.	72	2	n.a.	n.a.			

[1] – Except for % Moisture, all values are on a dry weight basis

[2] – Candle Barley, Lot # 04503, 31% pearled grain flour, 80% pass through a 250 *u*m screen

TABLE 2. Antibody profile of Chapters 3, tested in PBL [(species and clone number in brackets)mAb used in this assay were purchased from Serotec LTD. (Oxford, England) with the exception of CD3, CD8, CD25, and TCR, which were purchased from VMRD (Pullman, WA)].

Single Label Ab	**FITC** (Rabbit anti-Mouse IgG)	**TCR**[1] (Mouse 86D)	**CD8**[1] (Mouse PT81B)	**CD3**[1] (Mouse 8E6)	**Mono/Macro/Neut**[1] (Mouse MIL-2)	**B cell**[1] (Mouse Ig light chain K139 3E1)
Double Label Ab1	**CD4**[1] (Mouse MIL17)	**CD4**[1] (Mouse MIL17)	**CD4**[1] (Mouse MIL17)	**CD8**[1] (Mouse PT81B)	**CD8**[1] (Mouse PT81B)	**CD3**[1] (Mouse 8E6)
Double Label Ab2	**CD45Ra**[4] (Mouse MIL13)	**CD25**[4] (Mouse PGBL25A)	**CD8**[4] (Mouse PT81B)	**CD45Ra**[4] (Mouse MIL13)	**CD25**[4] (Mouse PGBL25A)	**TCR**[4] (Mouse 86D)

[1] Ab conjugated with F(ab')2 Rabbit anti mouse IgG:FITC STAR9B

[2] Ab conjugated with F(ab')$_2$ Rabbit anti rat IgG:FITC STAR17

[3] Ab conjugated with F(ab')$_2$ Rabbit anti rat IgG:FITC STAR17B

[4] Ab conjugated with F(ab')$_2$ Rabbit anti mouse IgG:RPE STAR12A

TABLE 3. Antibody profile of Chapters 4 and 5, tested in PBL, MLN and PP [(species and clone number in brackets)mAb used in this assay were purchased from Serotec LTD. (Oxford, England) with the exception of CD3, CD8, CD25, and Peyer's Patch B cell, which were purchased from VMRD (Pullman, WA)].

Peripheral Blood Lymphocytes

Single Label Ab	**FITC** (Rabbit anti-Mouse IgG)	**PE** (Rabbit anti-Mouse IgG)	**IgA**[1] (Mouse K61 1B4)	**IgM**[1] (Mouse K52-1C3)	**Mono/Macro/Neut**[1] (Mouse MIL-2)	**B cell**[1] (Mouse Ig light chain K139 3E1)	**CD25**[1] (Mouse PGBL25A)	**CD2**[2] (Rat MAC80)
Double Label Ab1	**CD2**[2] (Rat MAC80)	**CD44**[3] (Rat MAC329)	**CD3**[1] (Mouse 8E6)	**CD3**[1] (Mouse 8E6)	**CD44**[3] (Rat MAC329)		**CD4**[1] (Mouse MIL17)	**CD8**[1] (Mouse PT81B)
Double Label Ab2	**CD3**[4] (Mouse 8E6)	**CD3**[4] (Mouse 8E6)	**CD4**[4] (Mouse MIL17)	**CD8**[4] (Mouse PT81B)	**B cell**[4] (Mouse Ig light chain K139 3E1)		**CD45Ra**[4] (Mouse MIL13)	**CD45Ra**[4] (Mouse MIL13)

Mesenteric Lymph Nodes

Single Label Ab	**FITC** (Rabbit anti-Mouse IgG)	**B cell**[1] (Mouse Ig light chain K139 3E1)	**IgA**[1] (Mouse K61 1B4)	**IgM**[1] (Mouse K52-1C3)	**CD3**[1] (Mouse 8E6)	**CD4**[1] (Mouse MIL17)	**CD8**[1] (Mouse PT36B)
Double Label Ab1	**CD4**[1] (Mouse MIL17)	**CD8**[1] (Mouse PT81B)	**CD4**[1] (Mouse MIL17)		**CD8**[1] (Mouse PT81B)		**CD2**[2] (Rat MAC80)
Double Label Ab2	**CD45Ra**[4] (Mouse MIL13)	**CD45Ra**[4] (Mouse MIL13)	**CD25**[4] (Mouse PGBL25A)		**CD25**[4] (Mouse PGBL25A)		**CD3**[4] (Mouse 8E6)

Peyer's Patches

Single Label Ab	**FITC** (Rabbit anti-Mouse IgG)	**B cell**[1] (Mouse Ig light chain K139 3E1)	**PP B cell**[1] (Mouse BB6-10A10)	**IgA**[1] (Mouse K61 1B4)	**IgM**[1] (Mouse K52-1C3)	**Mono/Macro/Neut**[1] (Mouse MIL-2)	**CD2**[2] (Rat MAC80)	**CD3**[1] (Mouse 8E6)
Double Label Ab1	**CD44**[3] (Rat MAC329)			**CD4**[1] (Mouse MIL17)			**CD8**[1] (Mouse PT81B)	
Double Label Ab2	**B cell**[2] (Mouse Ig light chain K139 3E1)			**CD45Ra**[4] (Mouse MIL13)			**CD45Ra**[4] (Mouse MIL13)	

[1] Ab conjugated with F(ab')2 Rabbit anti mouse IgG:FITC STAR9B

[2] Ab conjugated with $F(ab')_2$ Rabbit anti rat IgG:FITC STAR17

[3] Ab conjugated with $F(ab')_2$ Rabbit anti rat IgG:FITC STAR17B

[4] Ab conjugated with $F(ab')_2$ Rabbit anti mouse IgG:RPE STAR12A

TABLE 4. Antibody profile of Chapters 6, tested in PBL, MLN and PP [(species and clone number given in brackets)mAb used in this assay were purchased from Serotec LTD. (Oxford, England) with the exception of CD3, CD25, and Peyer's Patch B cell, which were purchased from VMRD (Pullman, WA)].

Peripheral Blood Lymphocytes and Mesenteric Lymph Nodes						
Single Label Ab	**FITC** (Rabbit anti-Mouse IgG)	**IgM FITC** (Goat anti-Porcine IgM)	**PE** (Rabbit anti-Mouse IgG)	**IgA**[1] (Mouse K61 1B4)	**IgM**[1] (Mouse K52-1C3)	**Mono/Macro/Neut**[1] (Mouse MIL-2)
Single Label Ab	**CDw75**[2] (Mouse anti-Human LN-1)	**B cell**[1] (Mouse Ig light chain K139 3E1)	**CD3**[1] (Mouse 8E6)	**CD11b**[1] (Mouse MIL4)	**CD2**[3] (Rat MAC80)	
Double Label Ab1	**CD44:F** (Rat MAC329)	**CD25**[1] (Mouse PGBL25A)	**CD25**[1] (Mouse PGBL25A)	**CD45RA:F** (Mouse MIL13)	**CD45RA:F** (Mouse MIL13)	
Double Label Ab2	**CD3**[4] (Mouse 8E6)	**CD4:PE** (Mouse MIL17)	**CD8:PE** (Mouse MIL-12)	**CD4:PE** (Mouse MIL17)	**CD8:PE** (Mouse MIL-12)	
Peyer's Patches						
Single Label Ab	**FITC** (Rabbit anti-Mouse IgG)	**IgM FITC** (Goat anti-Porcine IgM)	**PE** (Rabbit anti-Mouse IgG)	**IgA**[1] (Mouse K61 1B4)	**IgM**[1] (Mouse K52-1C3)	**Mono/Macro/Neut**[1] (Mouse MIL-2)
Single Label Ab	**CDw75**[2] (Mouse anti-Human LN-1)	**B cell**[1] (Mouse Ig light chain K139 3E1)	**CD3**[1] (Mouse 8E6)	**CD11b**[1] (Mouse MIL4)	**PP B cell**[1] (Mouse BB6-10A10)	
Double Label Ab1	**CD44:F** (Rat MAC329)	**CD25**[1] (Mouse PGBL25A)	**CD25**[1] (Mouse PGBL25A)	**CD45RA:F** (Mouse MIL13)	**CD45RA:F** (Mouse MIL13)	
Double Label Ab2	**CD3**[4] (Mouse 8E6)	**CD4:PE** (Mouse MIL17)	**CD8:PE** (Mouse MIL-12)	**CD4:PE** (Mouse MIL17)	**CD8:PE** (Mouse MIL-12)	

[1] Ab conjugated with F(ab')2 Rabbit anti mouse IgG:FITC STAR9B

[2] Ab conjugated with Rat anti mouse IgM heavy chain:FITC

[3] Ab conjugated with $F(ab')_2$ Rabbit anti rat IgG:FITC STAR17

[4] Ab conjugated with $F(ab')_2$ Rabbit anti mouse IgG:RPE STAR12A

TABLE 5. Amino Acid composition of Teklad high protein casein (Cat. # 160030) used to formulate piglet diets.

Amino Acid	Percentage		Amino Acid	Percentage
Leucine	8.0		Alanine	2.7
Isoleucine	5.0		Arginine	3.3
Lysine	7.0		Aspartic Acid	6.1
Phenylalanine	4.4		Glutamic Acid	19.6
Threonine	3.8		Glycine	1.6
Tryptophan	1.0		Histidine	2.5
Valine	6.0		Proline	9.2
Methionine	2.3		Serine	4.8
Cystine	0.25		Tyrosine	4.6

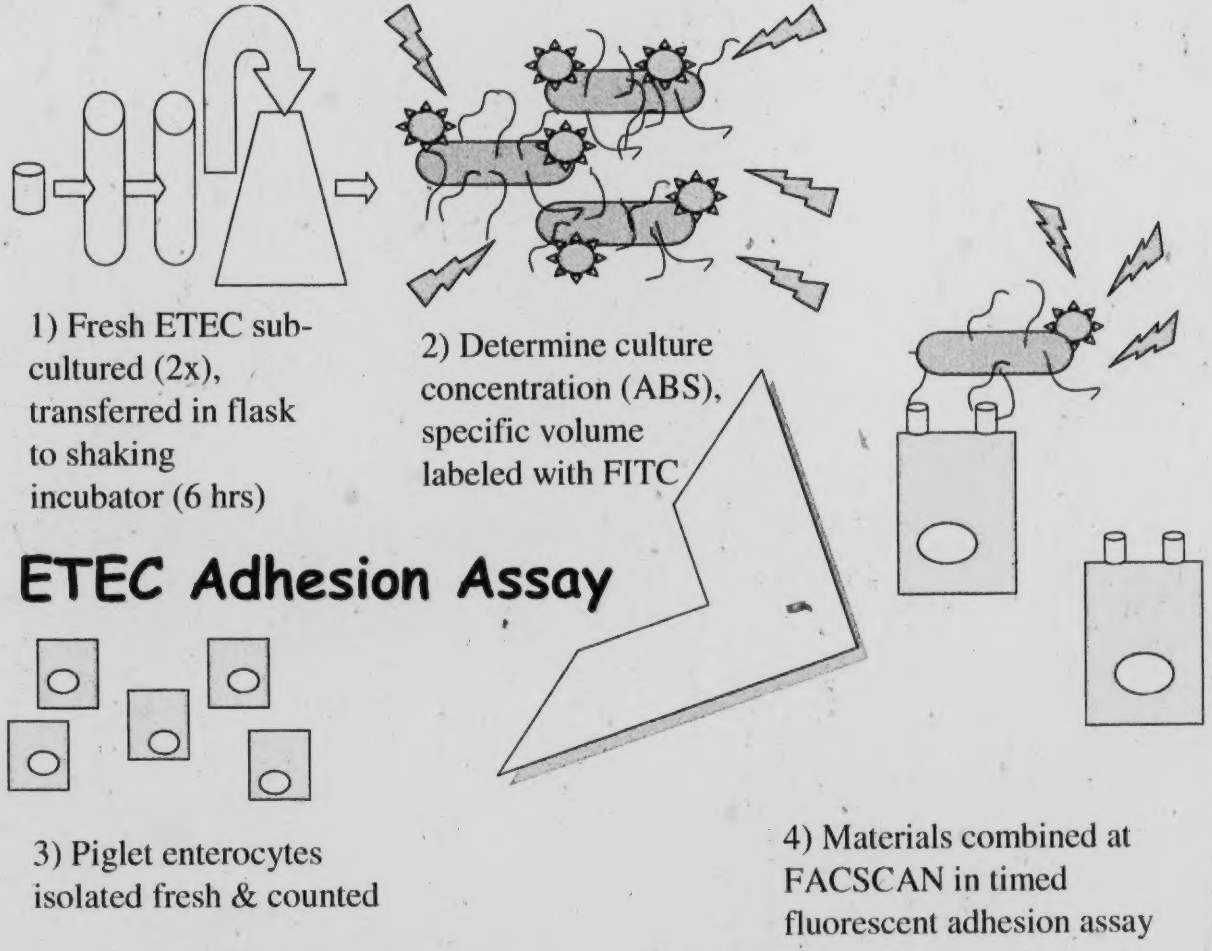
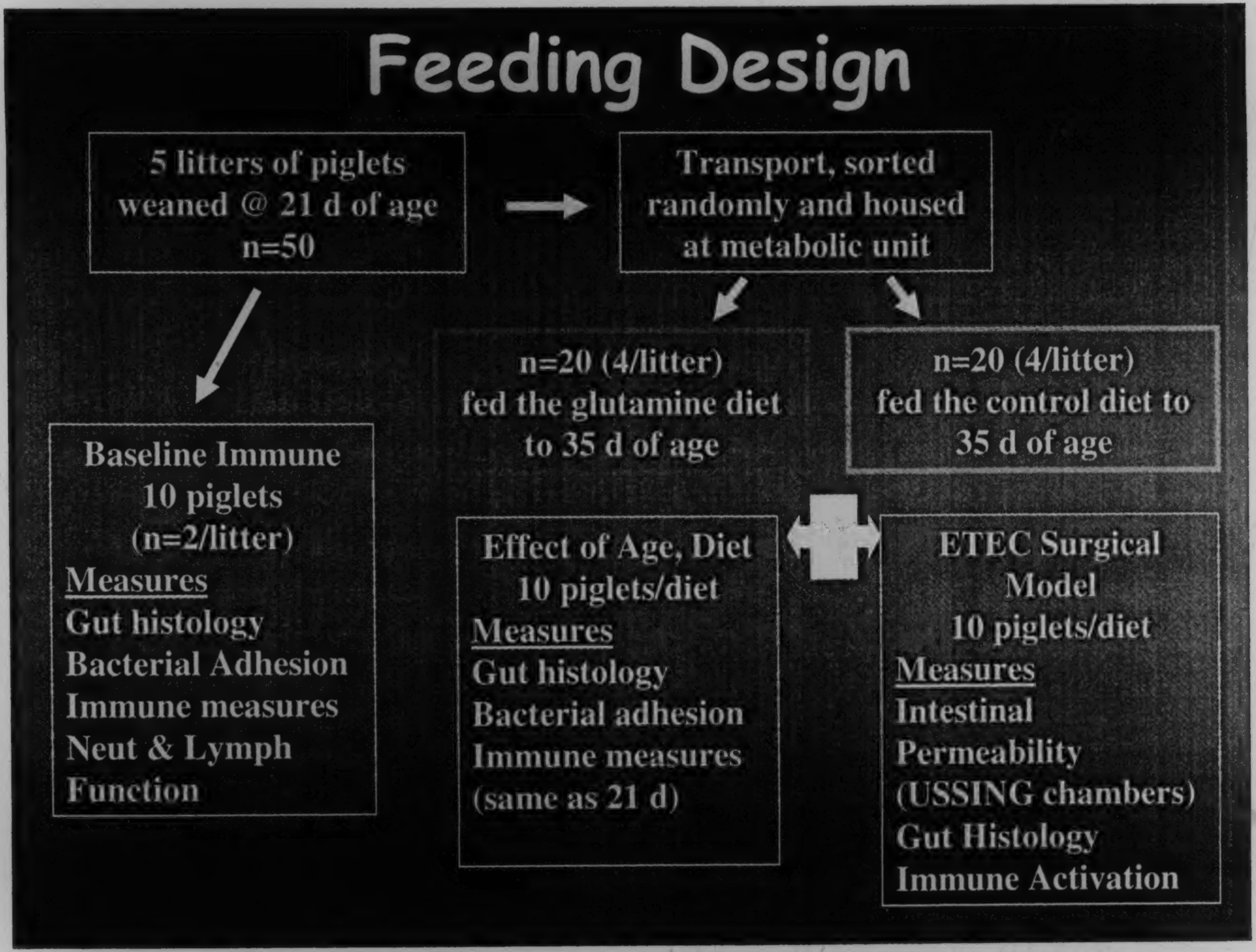

1) Fresh ETEC sub-cultured (2x), transferred in flask to shaking incubator (6 hrs)
2) Determine culture concentration (ABS), specific volume labeled with FITC
ETEC Adhesion Assay
3) Piglet enterocytes isolated fresh & counted
4) Materials combined at FACSCAN in timed fluorescent adhesion assay

Figure 1. Schematic representation of in vitro K88 ETEC Adhesion Assay



Feeding Design

5 litters of piglets
weaned @ 21 d of age
n=50

Transport, sorted
randomly and housed
at metabolic unit

n=20 (4/litter)
fed the glutamine diet
to 35 d of age

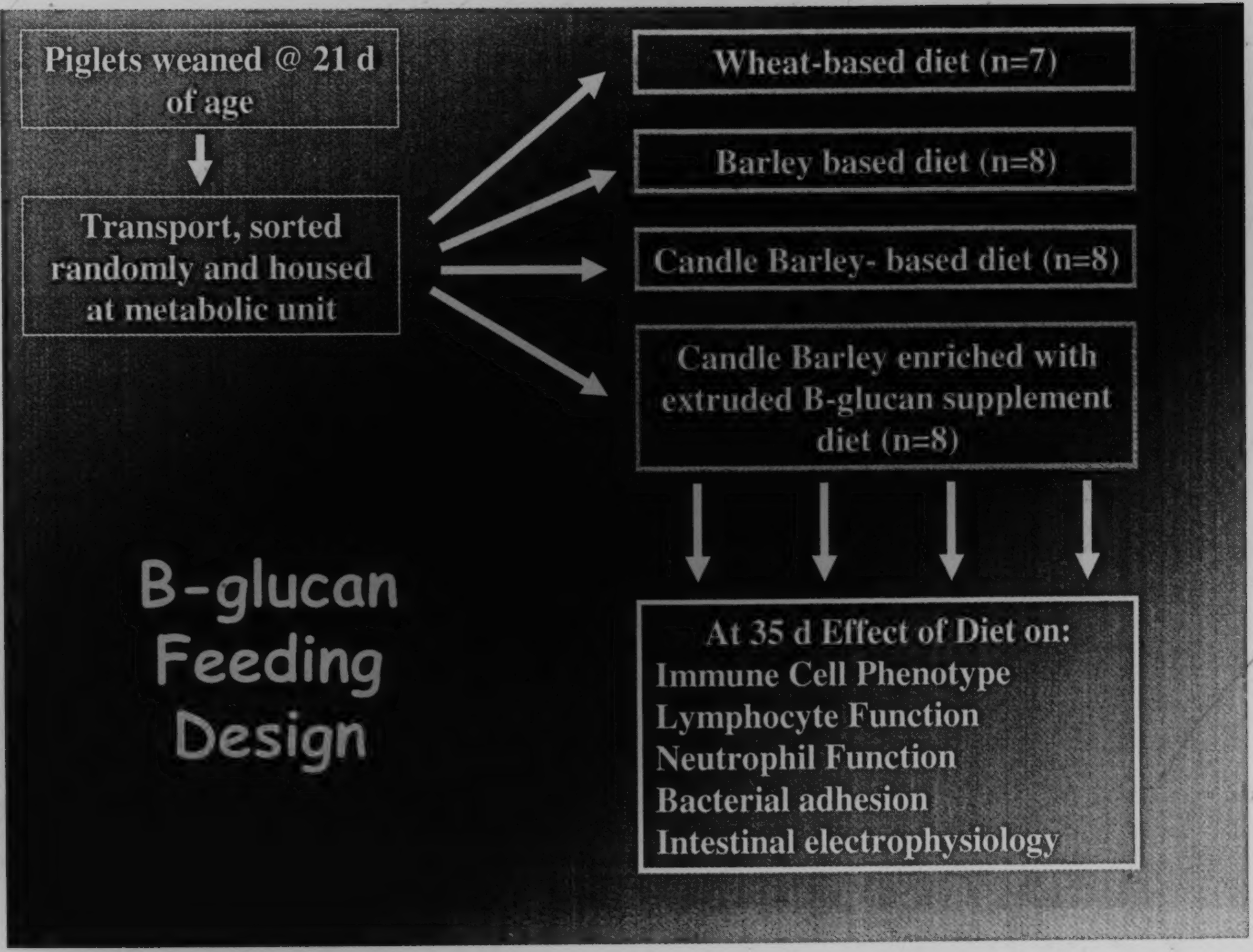
n=20 (4/litter)
fed the control diet to
35 d of age

Baseline Immune
10 piglets
(n=2/litter)
Measures
Gut histology
Bacterial Adhesion
Immune measures
Neut & Lymph
Function

Effect of Age, Diet
10 piglets/diet
Measures
Gut histology
Bacterial adhesion
Immune measures
(same as 21 d)

ETEC Surgical
Model
10 piglets/diet
Measures
Intestinal
Permeability
(USSING chambers)
Gut Histology
Immune Activation

Figure 2. Feeding Design for Chapter 4 – Influence of age and 5 – Influence of Glutamine



Piglets weaned @ 21 d of age

Transport, sorted randomly and housed at metabolic unit

Wheat-based diet (n=7)

Barley based diet (n=8)

Candle Barley- based diet (n=8)

Candle Barley enriched with extruded B-glucan supplement diet (n=8)

At 35 d Effect of Diet on:
Immune Cell Phenotype
Lymphocyte Function
Neutrophil Function
Bacterial adhesion
Intestinal electrophysiology

B-glucan Feeding Design

Figure 3. Feeding Design for Chapter 6 – Influence of dietary B-glucans

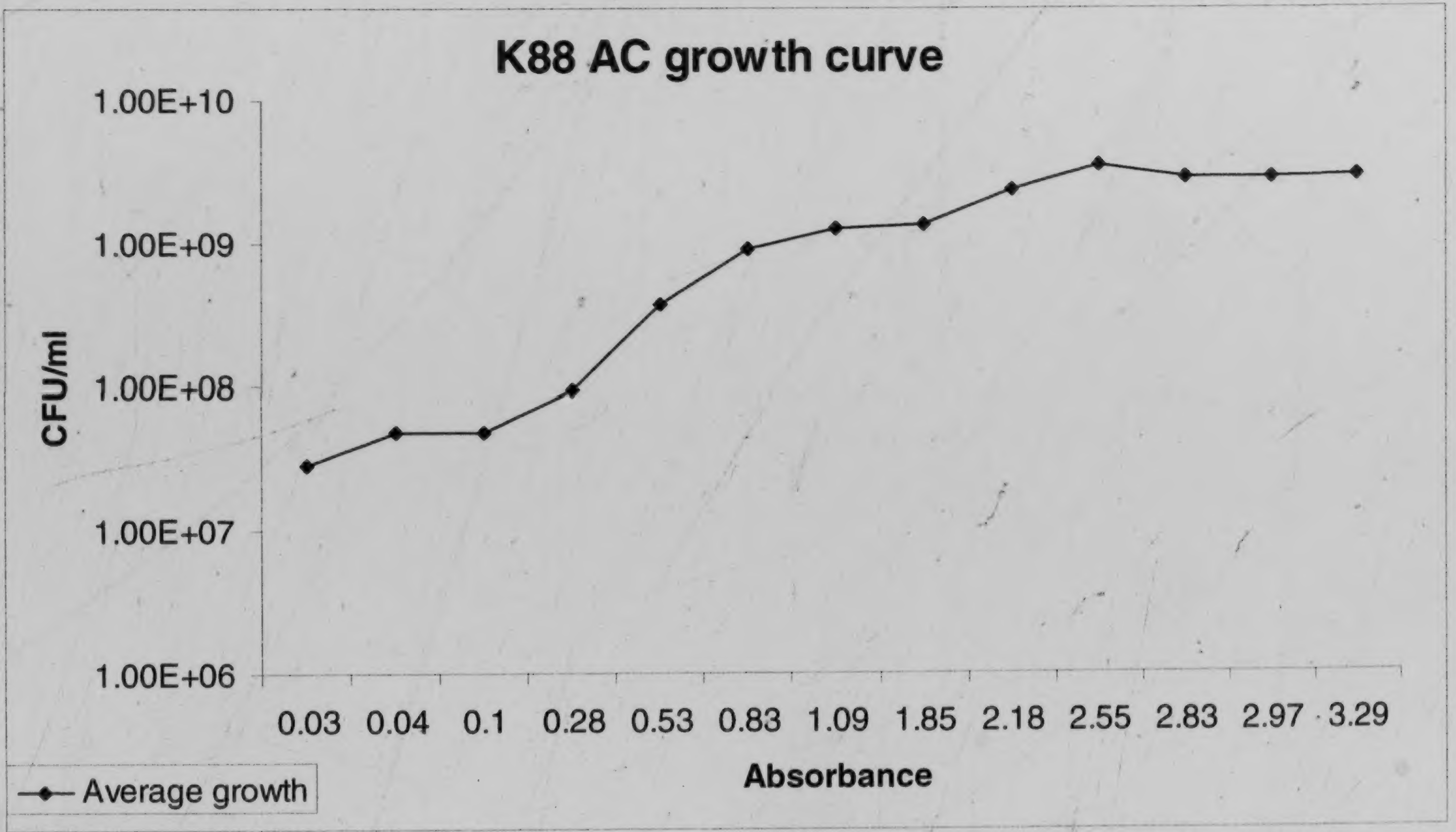


Figure 4. K88 ETEC Concentration Curve. The curve represents the mean values of two separate ETEC cultures (n=2) which were used in generating this curve. Samples were taken from the main culture at 30 min intervals and the Absorbance of each sample was determined. Plate counts were performed on each of the timed samples. The amount of ETEC (CFU/ml) was then plotted versus the Absorbance. The concentration of subsequent cultures of ETEC was determined by reading absorbance and finding the corresponding concentration from the curve.